Pancreatic Head Adenocarcinomas -

Histopathologic Evaluation and Prognostic Factors

Ewa Pomianowska

Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Norway

&

Department of Hepato-Pancreato-Biliary Surgery

Department of Pathology

Department of Pharmacology

Oslo University Hospital, Rikshospitalet, Norway

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2. Abbreviations and definitions

AA	Arachidonic acid
AC	Ampullary cancer
AJCC	American Joint Committee of Cancer Staging
αSMA	α- smooth muscle actin
EGF	Epidermal growth factor
cAMP	Cyclic adenosine monophosphate
COX-2	Cyclooxygenase type 2
DBC	Distal bile duct cancer
DC	Duodenal cancer
ECM	Extracellular matrix
IHC	Immunohistochemistry
IL	Interleukin
LNR	Lymph node ratio
PC	Pancreatic cancer
PDGF	Platelet-derived growth factor
PGE_2	Prostaglandin E_2
PSC	Pancreatic stellate cells
TGFβ	Transforming growth factor beta
UICC	Union for International Cancer Control
WHO	World Health Organisation

3. List of publications

1. Pomianowska E, Grzyb K, Westgaard A, Clausen OPF, Gladhaug IP

Reclassification of tumour origin in resected periampullary adenocarcinomas reveals underestimation of distal bile duct cancer

Eur J Surg Oncol 2012; 38:1043-1050.

2. Pomianowska E, Westgaard A, Mathisen Ø, Clausen OPF, Gladhaug IP

Prognostic relevance of number and ratio of metastatic lymph nodes in resected pancreatic, ampullary and distal bile duct carcinomas

Ann Surg Oncol 2013; 20:233-241

3. Pomianowska E, Schjølberg AR, Clausen OPF, Gladhaug IP

COX-2 overexpression in resected pancreatic head adenocarcinomas correlates with favorable prognosis

BMC Cancer 2014; 14:458

4. Pomianowska E *, Sandnes D*, Grzyb K, Schjølberg AR, Aasrum M, Tveteraas IH, Tjomsland V, Christoffersen T, Gladhaug IP

Inhibitory effects of prostaglandin E2 on collagen synthesis and cell proliferation in human stellate cells from pancreatic head adenocarcinoma.

BMC Cancer 2014; 14:413

***** These authors contributed equally

4. Summary

Primary adenocarcinomas located in the pancreatic head may arise from the distal bile duct, the ampulla, the periampullary duodenum, and the pancreatic tissue itself. These tumours are distinct cancer entities whose pathobiology, staging, and clinical course have unique features.

The aims of the present study were to investigate how the standardized histopathological examination protocol ensures correct classification of tumour origin of pancreatic head adenocarcinomas. Furthermore, we examined how different approaches to assessment of lymph node metastases and expression of cyclooxygenase-2 (COX-2) in the tumours can predict long-term outcome. Finally, we assessed COX-2 expression and effects of PGE_2 on cells proliferation and collagen synthesis in pancreatic stellate cells in vitro.

The precise anatomical site of origin is often difficult to establish during preoperative investigations or during surgery and therefore the final diagnosis is always the result of the histopathological examination. The predetermined diagnostic criteria, with special focus on the anatomical site of origin, are essential to improve the accuracy of diagnosis. Furthermore, high workload per pathologist increases the precision of the histopathologic diagnosis.

The presence of lymph node metastasis is a major determinant of long-term survival in pancreatic head cancers. In patients operated with pancreatoduodenectomy, N status and LNR are superior to the number of metastatic node as prognostics indicators. The predictive value of these variables depends on the cancer origin. In ampullary and distal bile duct cancer, N status discriminates between subgroups of patients with different long-term survival whereas in pancreatic cancer, LNR is clearly more powerful in prognostic subclassification. In patients with pancreatic cancer, multivariate analysis identified LNR > 0.2 as an independent predictor of poor long-term survival. LNR could therefore be proposed as a standard, alternative measure of nodal involvement in the pancreatic cancer.

Overexpression of COX-2 has been described in several tumours, however the data on the prognostic importance of COX-2 in pancreatic head adenocarcinomas are inconsistent. In our study COX-2 is overexpressed in >70% of pancreatic, ampullary and distal bile duct cancers and is associated with the histopathological type of differentiation, with the degree of differentiation, and with a favourable prognosis. In pancreatic cancer, in a multivariate model, COX-2 negative tumours and LNR > 0.2, independently predicted poor prognosis.

When assessed by immunohistochemistry, COX-2 is mainly expressed in pancreatic carcinoma cells, and these cells are regarded as the main source of PGE_2 in pancreatic cancer tumour tissue. COX-2 was not detected in the stroma, however COX-2 was detected in the cultured pancreatic stellate cells (PSC), and could be further induced by interleukin-1β (IL-1β), epidermal growth factor (EGF), thrombin, and PGE_2, but not by transforming growth factor-β1 (TGFβ). Treatment of PSC with PGE_2 suppressed both TGFβ-stimulated collagen synthesis and PDGF-stimulated DNA synthesis, suggesting that inhibition of COX-2 may inadvertently accelerate fibrosis progression in pancreatic cancer.

This thesis confirms that standardised histopathological evaluation after pancreatoduodenectomy with special focus on the tumour origin, lymph node assessment, degree and type of differentiation is necessary to obtain accurate and reliable survival estimates. Furthermore, our findings of the COX-2 expression in the tumours, and the PGE_2 effects on pancreatic stellate cells, revealed new aspects of biological mechanisms involved in progression of pancreatic cancer.

5. Introduction

5.1 The pancreatic gland

The pancreas is a glandular organ, oval-shaped, located retroperitoneally on the posterior upper part of the abdomen, behind the stomach, across the lumbar (L1-2) spine (Fig.1). The pancreatic head constitutes about two- thirds of the pancreatic volume and the body (corpus) and tail (cauda) make up the remaining of the pancreatic parenchymal mass (Fig.2). The head of the pancreas lays in the duodenal loop in front of the inferior vena cava (IVC) and the left renal vein. The uncinate process is an extension of the inferior half of the head toward the left; it is of varying size and is wedged between the superior mesenteric vessels (vein and artery) in front and the aorta behind it. The body and tail of the pancreas run obliquely upward to the left in front of the aorta and left kidney. The narrow tip of the tail of the pancreas reaches the splenic hilum in the splenorenal (lienorenal) ligament. The pancreatic neck is the arbitrary junction between the head and body of the pancreas, with the portal vein immediately adjacent behind.

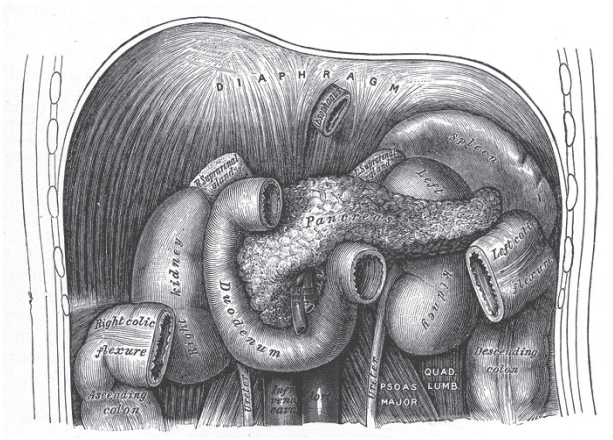

Figure 1
The duodenum and pancreas. Henry Gray. Anatomy of the Human Body, 1918 (copyright expired)

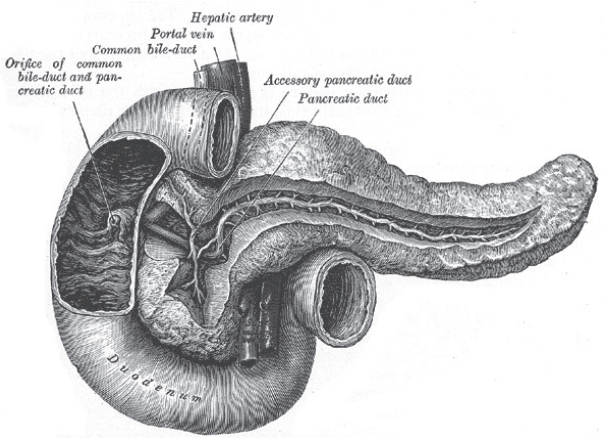

Figure 2
The pancreatic gland. Henry Gray. Anatomy of the Human Body, 1918 (copyright expired)

The pancreas is classified as a heterocrine gland because it contains both endocrine and exocrine glandular tissue. Most of the pancreatic tissue mass (80-90%) is composed of exocrine tissue. The endocrine portion of the pancreas is composed of small clusters of cells called islets of Langerhans, which are scattered throughout the exocrine tissue. There are two main types of endocrine cells that make up the islets: alpha cells and beta cells. Both types are involved in glucose homeostasis - alpha cells produce glucagon and beta cells produce insulin. The exocrine tissue is arranged into many small masses known as *acini*. Acini are small grapes -like clusters of exocrine cells that surround tiny ducts. The exocrine cells in the acini produce digestive enzymes in response to stimuli from the stomach and the duodenum[1]. After the enzymes are secreted from the cells, they enter the ducts, where ductal cells add mucous and bicarbonate to the enzyme mixture. The ducts of many acini connect to form larger ducts culminating in the major pancreatic duct of Wirsung, which empty into the duodenum at the papilla of Vater (Fig.2). In some anatomical variants the main and accessory pancreatic ducts failure to fuse and form pancreas divisum where the accessory pancreatic duct (of Santorini) drains the body and tail of pancreas via the additional minor papilla into the duodenum.

The common distal bile duct enters the pancreatic gland from behind and joins the main pancreatic duct, forming the ampulla of Vater. There are a number of variations in the anatomy of the pancreatic ductal system and their relationship to the common biliary duct[2]. Usually, there is a short common channel –which forms ampulla of Vater. However, in some cases, the major pancreatic duct and the common bile duct do not fuse and enter the duodenum thorugh two separate openings. In those cases, the ampulla of Vater, as a separate anatomical site, does not exist.

5.2 Pancreatic head adenocarcinomas

Primary adenocarcinomas located in the pancreatic head may arise from the pancreatic tissue itself, the ampulla of Vater, the distal bile duct and the periampullary duodenum. These adenocarcinomas have different biological and pathological features, as well as clinical course and overall prognosis[3]. These adenocarcinomas have often been collectively named periampullary carcinomas. Although the term *periampullary carcinoma* might be a good term to define this heterogeneous group of neoplasms, there is substantial inconsistency in the published literature regarding its definition[4]. Most often, in the surgical literature, this term is referred to carcinomas which can be removed by pancreatoduodenectomy, irrespectively of the tumour origin (duodenal, ampullary, distal bile duct or pancreatic)[5;6]. However, in other publications, this term might be restricted only to tumours arising from the ampulla of Vater or/and duodenum[7]. Others defined it as tumours arise near (e.g. < 2 cm) the papilla in the duodenum, thus mostly excluding tumours originating in the pancreatic tissue itself [8].

Since the disagreement in terminology is considerable, and to avoid further confusion, we decided to completely avoid the term *periampullary tumours.* In the present thesis adenocarcinomas originating from the ampulla of Vater, the distal bile duct, the periampullary duodenum and the pancreatic head tissue itself are denoted *pancreatic head adenocarcinomas*.

5.2.2 Pancreatic cancer

Pancreatic adenocarcinoma is the fourth leading cause of cancer death in men and woman in western societies, with 5-year survival of less than 5% [9]. The outcome of pancreatic cancer has remained largely unchanged for over the last three decades despite vast improvement in understanding of the biological and the clinical aspects of the disease, and improvement in the surgical and medical care. Adenocarcinoma of the pancreas is characterized by a high prevalence of genetic alterations, a propensity to metastasize at a very early stage, and a general resistance to conventional chemotherapy and radiation therapy[10;11].

Molecular studies on pancreatic adenocarcinomas have revealed a number of genetic alterations, which involve activation of K-ras, inactivation of p53 and p16, and deregulations of growth factors or growth factor receptors[12]. The genetic alterations have also been identified in precursor lesions of pancreatic cancer[12;13]. The transformation of normal duct epithelial cells into invasive adenocarcinoma is believed to be gradually developed through formation of lesions that initiates diverse changes in the morphology and functions of the cells. The most common neoplastic precursors to invasive adenocarcinoma are pancreatic intraepithelial neoplasia (PanIN), mucinous

cystic neoplasm (MCN) and intra-ductal papillary mucinous neoplasm (IPMN). PanIN development may be separated into 3 stages, beginning with ductal cell hyperplasia (PanIN-1A and 1B), followed by atypical hyperplasia (Pan IN-2), and the formation of high grade dysplasia (PanIN-3)[14;15]. The latter is frequently present adjacent to pancreatic adenocarcinoma on histopathological examination of resected specimens. MCNs are composed of mucin- producing epithelial cells with a distinctive ovarian type stroma and may also progress to invasive adenocarcinoma[16]. IPMNs are large mucin-secreting neoplasms, which may progress to either mucinous non – cystic (colloid) carcinoma or ductal adenocarcinoma[13;17;18].

The incidence of pancreatic cancer is strongly age-dependent. The peak incidence of the disease is in the seventh to eighth decades of life, and most cases (80%) occur between the ages 60 and 80 years. Cigarette smoking has been consistently identified as one of the strongest risk factors, which is thought to be involvement in as much as 15-30% of all cases[19;20]. A number of dietary factors are also considered associated with pancreatic cancer. High intake of fats, nitrates, meat, dairy products and high total energy intake and general obesity (BMI $\geq$30 kg/m^2) have all been shown to be associated with increased risk of PC[21]. Furthermore, it is generally accepted that patients with longstanding diabetes mellitus (type II), have a two-fold increased risk of developing PC[22].

Results from the studies which examined alcohol consumption, coffee habits and chronic pancreatitis as possible risk factors have been contradictory and inconclusive[19;23]. Regular physical activity, diets high in fruits, vegetables, fiber and vitamin C appear to decrease the risk of developing PC[21;24;25].

In approximately 5-to10 percent of pancreatic cancer, various genetic factors are thought to be associated with the disease. The genetic disorders associated with an increased risk of pancreatic cancer include hereditary breast and ovarian cancer syndrome (BRCA-2), hereditary pancreatitis, familial atypical multiple mole melanoma (FAMMM) syndrome, Peutz-Jeghers syndrome, hereditary nonpolyposis colorectal cancer syndrome (HNPCC), and Li-Fraumeni syndrome[14;26]. However, these genetic disorders account for very few of the total number of patients who develop PC.

5.2.3 Distal bile duct cancer

Cholangiocarcinomas, as a whole, accounts for approximately 3% of all gastrointestinal cancers. Distal bile duct tumours arise between the junction of the cystic duct- bile duct and the ampulla of Vater, and account for 20% to 30% of all cholangiocarcinomas[27,28]. The likelihood of developing distal cholangiocarcinoma increases with age, with a peak in the seventh decade. There also tends to be a slight male predominance for these lesions.

These tumours originate from cholangiocytes, the epithelial cell lining the bile ducts[29]. Most cases of cholangiocarcinomas are sporadic and without an identifiable cause. However, there are some

inflammatory condition involving the bile ducts, like primary sclerosing cholangitis (PSC)[30], chronic stone disease[31], choledochal cysts, Caroli disease and diverse infectious which may also initiate the development of these tumours.
Recently, precursors lesions associated with development of cholangiocarcinoma have been described[32;33]. The main types are biliary intraepithelial neoplasia (BilIN) 1-3 and intraductal papillary neoplasm of the bile duct (biliary IPN).

5.2.4 Ampullary cancer

Adenocarcinomas in the ampulla of Vater are not very common, representing only 0.2% of all gastro-intestinal cancers and accounting for 15-25% of neoplasms arising in the periampullary region[5;6;34-39]. These tumours typically have either intestinal or pancreatobiliary-type of histologic differentiation[40;41]. Most carcinomas of the ampulla of Vater develop sporadically, however they are often associated with pre-existing ampullary adenomas[42]. The peak age incidence of sporadic ampullary carcinomas is in the seventh and eighth decades of life and males appear to be more affected than females. Cigarette smoking has been discussed as a risk factor for development of this malignancy. Similarly to pancreatic and distal cholangiocarcinomas, ampullary carcinomas are also associated with several genetic alteration[43-45].

5.2.5 Periampullary duodenal cancer

Primary malignant tumours of the duodenum represent 0.3% of all gastro-intestinal tract tumours but up to 50% of small bowel malignancies. The large majority of duodenal tumours are adenocarcinomas[46]. The tumours can be located in any part of the duodenum but the most frequent location is the descending part.

The major duodenal papilla is situated in the second part of duodenum, about 7-10 cm from the pylorus. It is surrounded by the sphincter of Oddi, and receives a mixture of pancreatic enzymes and bile from the Ampulla of Vater which drains both the pancreatic and distal bile duct.

The major duodenal papilla is seen from the duodenum as lying within a mucosal fold. The minor duodenal papilla is situated 2 cm proximal. Adenocarcinomas arising from the duodenal papilla are sometimes mistakenly regarded as the amullary carcinomas, while they represent the pure duodenal tumours [47].

Adenocarcinoma of the duodenum may arise from duodenal polyps observed in familial polyposis or Gardener's syndrome, or be associated with celiac disease[48;49]. The 5-year survival rate varies widely

according to the series published, but is generally reported to be > 40% in case of curative resection. The peak of frequency is the sixth decade, although the disease may develop in younger patients.

5.3 Treatment

5.3.1 Surgical treatment

Due to the complexity of the regional anatomy pancreatic, distal bile duct, ampullary and periampullary duodenal cancers are all resected by a common surgical procedure. Surgical resection is the only potentially curative therapy for most of those tumours; however, resection is frequently not possible due to locally advanced disease or distant metastasis.

Thus at the time of diagnosis, only 10 to 20% of the tumours of the pancreatic head are surgically resectable and the number is even lower when the carcinoma is situated in the body or tail of the gland.

The treatment of choice for a pancreatic head tumour is a pancreatoduodenectomy, which can be performed as classical[50] or pylorus-preserving[51]. The classical pancreatoduodenectomy, often referred to as a Whipple's procedure, involves surgical removal of the pancreatic head, duodenum, the distal bile duct, the gallbladder, and the distal part of the stomach. In the pylorus-preserving pancreatoduodenectomy, the distal part of the stomach with pylorus is not removed. Both procedures are performed with similar morbidity and mortality[52].

The extend of lymphadenectomy during pancreatoduodenectomy has been a matter of discussion. Lymphadenectomy can be carried out as a standard or an extended procedure. Currently a standard procedure, including removal of the lymph nodes of the right side of the hepatoduodenal ligament, anterior and posterior pancreaticoduodenal nodes and nodes to the right side of the superior mesenteric artery as *en bloc* resection is recommended. Additional nodes of the anterior- superior region of the common hepatic artery are removed separately [53] (Fig.3). Radical or extended lymphadenectomy includes removal of the glands near the common and proper hepatic artery, celiac axis, right and left side of the hepatoduodenal ligament, around superior mesenteric artery and the inferior pancreaticoduodenal artery, and all lymph nodes of the anterolateral aspect of the aorta and of the inferior vena cava (Fig.3). Extended lymphadenectomy is currently not recommended since no survival benefit is obtained and the complications rates are higher compared to standard lymphadenectomy[54-56].

Tumours located in body and tail of the pancreas are removed by a distal pancreatic resection. Regional lymphadenectomy with *en bloc* resection of the nodes along celiac axis, splenic artery, and

splenic hilum and along the inferior border of the body and tail of the pancreas, is recommended in these cases[53].

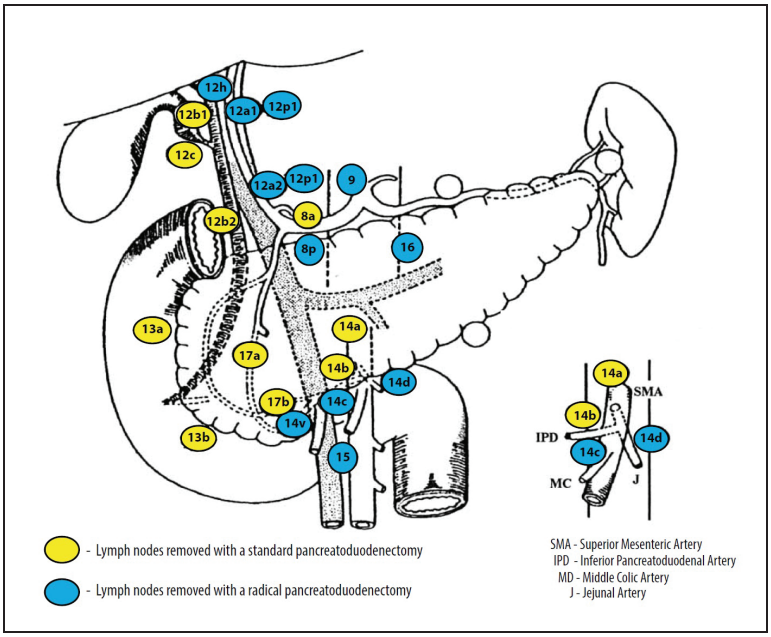

Figure 3
Lymph nodes removed with a standard (yellow circle), or radical (blue circle) pancreatoduodenectomy. Modified from Kawarada et.a[57]*l.*

5.3.2 Medical treatment

In the majority of patient with pancreatic cancer, curative surgery is not an option, and chemotherapy remains the only treatment. Unfortunately, currently available chemotherapy in advanced pancreatic caner has very limited effect on disease survival and is primarily aimed at palliating symptoms to ensure better quality of life[58].

Two chemotherapies are mainly used as the adjuvant and palliative treatment for the patients with pancreatic cancer, i.e. 5-Fluorouracil (5-FU) or Gemcitabine. Since its approval in 1997, Gemcitabine was found superior to 5-FU, and has been the standard first –line palliative treatment worldwide for patients with PC[59].

Until December 2007, national guidelines for pancreatic cancer in Norway did not recommend adjuvant treatment. As from 2008, adjuvant therapy with 5-Fluorouracil is recommended for eligible patients which are operated for pancreatic cancer with curative intent, regardless of R-status or N-stage [60].

For the two other tumour types removed with pancreatoduodenectomy, ampullary and distal bile duct cancer, there are no national guidelines regarding adjuvant treatment after surgery. In the cases where lymph node metastasis are detected and /or R1 resection status is present, adjuvant chemotherapy, similar to chemotherapy in the pancreatic cancer, might be considered. Patients with duodenal cancer usually receive the same type of chemotherapy that is recommended for patients with colorectal adenocarcinoma[61].

5.4 Prognosis

Table 1. The 5-year survival rate for patients operated with pancreatoduodenectomy for pancreatic, distale bile duct, ampullary and duodenal adenocarcinoma

Pancreatic cancer[5;38;62-65]	Distal Bile Duct Cancer[5;38;62-65]	Ampullary cancer[5;38;62-65]	Duodenum cancer[5;38;65]
5-20%	**13-53%**	**35-54%**	**44-59%**

Long-term survival after pancretoduodenectomy varies substantially among adenocarcinomas derived from pancreas, distal bile duct, ampulla and duodenum (Table 1). There is also considerable variation in survival data within the individual tumour sites as reported in different series of pancreatoduodenectomy from European and American centres. It is especially prominent in pancreatic cancer where the 5-year survival varies from five to twenty percent. Although this variation might be at least partly explained by differences in selection of patients or dissimilar approach to surgical management, it also indicates problems with correct histopathological assessment of tumour origin. Tumours with “better prognosis” originating in Ampulla of Vater or duodenum might be mistakenly diagnosed as pancreatic cancer and thus causes a falsely favourable prognosis for pancreatic cancer in the survival analysis.

5.5 TNM classification

The classification of tumours in the pancreatic head removed by pancreatoduodenectomy is based on the TNM system, revised in 2010[66] (Table 2). In pancreatic cancer, the definition of T1 and T2 -status is linked directly to the size of the tumour within pancreas. In ampullary and distal bile duct invasion to the adjacent structures signifies T2 status. The pancreatic cancers however, regardless of size, are

classified as T3 tumours when they extend beyond the pancreas without affecting the celiac trunk or the superior mesenteric artery.

Table 2. TNM classfication 2010[61]

	Pancreatic cancer	Distal bile duct cancer	Ampullary cancer	Duodenal cancer
T-status				
T1	Tumour limited to pancreas, 2 cm or less in dimension	Tumour confined to the bile duct histologically	Tumour limited to ampulla of Vater of sfincter Oddi	Tumour invades lamina propria or/and submucosa
T2	Tumour limited to pancreas, more than 2 cm in dimension	Tumour invades beyond the wall of the bilde duct	Tumour invades duodenal wall	Tumour invades muscularis propria
T3	Tumour extends beyond pancreas but without involvement of CA or AMS	Tumour invades gallbldder, pancreas, duodenum or othe adjacent organs without involvement of CA of AMS	Tumour invades pancreas	Tumour invades through the muscularis propria with extension 2 cm or less
T4	Tumour inviolves CA or AMS (unresectable primary tumour)	Tumour involves CA or AMS	Tumour invades peripancreatic soft tissues or other adjacent organs or structures other than pancreas	Tumour perforates the visceral peritoneum with extension more than 2 cm and/or invades pancreas or bile duct
N-status				
N0	No regional node metastasis	No regional node metastasis	No regional node metastasis	No regional node metastasis
N1	Regional node metastasis	Regional node metastasis	Regional node metastasis	Metastasis in 1to 3 regional lymph nodes
N2				Metastasis in 4 or more regional lymph nodes
M-status				
M0	No distant metastasis	No distant metastasis	No distant metastasis	No distant metastasis
M1	Distant metastasis	Distant metastasis	Distant metastasis	Distant metastasis

CA - celiac axis
AMS - arteria mesenteric superior

5.6 Lymph node status

TNM staging forms the basis for estimation of the prognosis of gastrointestinal epithelial malignancies. In most studies, tumour size and the nodal status are the most important predictors of prognosis. According to current AJCC guidelines, adequate staging of node-negative pancreatic cancer requires evaluation of minimum 12 lymph nodes, although N0 is assigned even when this number is not met as long as all evaluated lymph nodes are found negative[67].

Recent publications have called attention to the ratio of positive (metastatic lymph nodes) to resected lymph nodes (total yield of resected nodes) as a negative prognostic factor[68]. The ratio of these two values is called the lymph node ratio (LNR). LNR may improve discrimination between prognostic groups by taking into account the extent of metastatic disease (number of positive nodes), as well as

the adequacy of lymphadenectomy and its histopathologic analysis (total number of nodes removed and identified in the surgical specimen). As LNR may to some degree compensate for variations in surgical and pathological node dissections it has therefore been proposed as an alternative measure of nodal involvement in adenocarcinomas of the pancreatic head[68].

5.7 Stellate cells, tumour stroma

A particular feature of primary pancreatic adenocarcinoma is the extensive fibrotic stromal reaction surrounding these tumours, known as tumour desmoplasia. Numerous lines of evidence suggest that pancreatic stellate cells (PSC) have a major role in the development of fibrosis associated with chronic pancreatitis and pancreatic cancer[69-72].

PSC are resident cells in the exocrine pancreas, comprising approximately 4-7% of the total cell mass of the gland[73;74]. The cells are located adjacent to the basolateral aspect of pancreatic acinary cells. In a healthy gland, PSC are in their quiescent state, characterised by abundant vitamin A storing lipid droplets in the cytoplasm and showing a "star" shaped morphology[73]. They can be differentiated from fibroblasts due to expression of desmin, glial fibrillary acidic protein (GFAP), nestin and neural cell adhesion molecule. PSC play an important role in normal extracellular matrix (ECM) turnover via their ability to both synthesize and degrading matrix molecules[69-72].

Upon activation, in response to injury, inflammation, or in culture, the PSC loose their vitamin A droplets and assume a myofibroblasts-like phenotype, express the cytoskeletal protein α-smooth muscle actin (αSMA) and synthesize excessive amounts of extracellular matrix proteins leading to fibrosis (Fig. 4).

a. b.

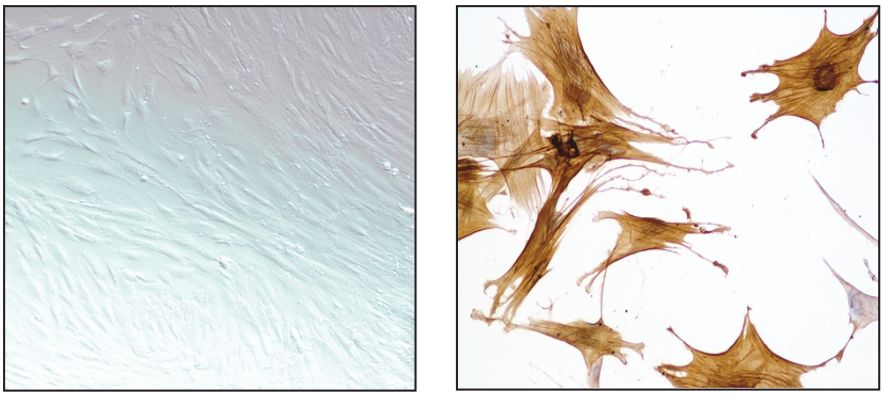

Figure 4. Activated pancreatic stellate cells in culture. a. large amount of extracellular matrix proteins b. expression of the cytoskeletal protein α-smooth muscle actin (αSMA)

Histological and immunohistochemical studies of human pancreatic cancer have shown that activated PSC are present and are responsible for producing the stromal reaction in the desmoplastic areas of pancreatic cancer[75]. Studies of human and rat PSC in culture have identified a number of growth factors, cytokines, and hormones as regulators of pancreatic stellate cell activation [70]. Platelet-derived growth factor (PDGF)[76], transforming growth factor-ß (TGFß)[70;77], interleukin -1 ß (Il-1ß)[78] and epidermal growth factor (EGF)[79] are some of the factors which regulate proliferation, activation, collagen production and apoptosis in the pancreatic stellate cell.

The effects of cyclooxygenase -2 (COX-2) and its product prostaglandin-E_2 (PGE_2) are widely studied in fibroblasts, mainly in lung and liver. In the lung, PGE_2 has the ability to limit fibroblast proliferation and migration and has been found to inhibit collagen synthesis by activating EP2 receptors and stimulating cAMP accumulation[80]. In patients with idiopathic pulmonary fibrosis, lung fibroblasts display low capacity to express COX-2 and to synthesize PGE_2 resulting in fibroblast accumulation and relentless deposition of collagen[81-83]. In the liver, PGE_2 via the cAMP pathway negatively regulates the proliferative effect of growth factors on hepatic stellate cells[84]. Thrombin and PDGF stimulate the release of PGE_2, which again exerts an inhibitory effect on DNA synthesis induced by PDGF and thrombin. Although the functions of prostaglandin E_2 (PGE_2) are well defined in lung and liver, its influence on the functions of the pancreatic stellate cells, particularly the effects on pancreatic fibrosis and cell proliferation, is still uncertain.

Several lines of evidence suggest that in pancreatic cancer, PSC are of great importance for tumour progression, by interacting in many ways with the malignant cells, such as reciprocal paracrine proliferative stimulation and angiogenesis, contributing to the early invasive growth and metastasis of this tumour[70;71]. Coculture experiments with PSC and pancreatic cancer cell lines confirm these findings, showing that exposure of PSC to cancer cells either directly or via conditioned media results in activation of PSC and consequently increased proliferation, ECM synthesis and migration[71]. In turn, PSC stimulate cancer cell proliferation and inhibit cancer cell apoptosis[85] (Fig. 5).

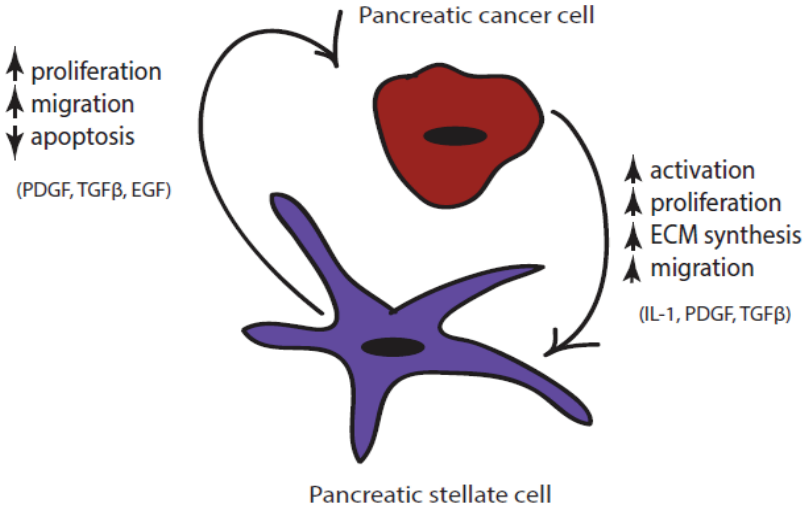

Figure 5. Bidirectional interactions between pancreatic cancer cells and stellate cells

5.8 COX-2

Cyclooxygenase (COX) is a rate limiting enzyme involved in the conversion of arachidonic acid into prostaglandin H_2, which represents a precursor of several bioactive molecules, including prostaglandin E_2, prostacyclin, and tromboxane[86] (Fig. 6). Two different isoenzymes, COX-1 and COX-2, have been identified[87]. There has also been reported a third isoform of COX enzyme, COX-3, which is a splice variant of COX-1, thus by some named COX-1b or COX-1 *variant* (COX-1v)[88]. COX-1 is constitutively expressed in many tissues including kidney, lung, stomach, duodenum, jejunum, ileum, colon and coecum[89]. COX-1 exerts diverse physiological functions such as maintaining gastric mucosa, platelet aggregation, maintenance of renal blood flow, glomerular filtration and ovulation[90,91].

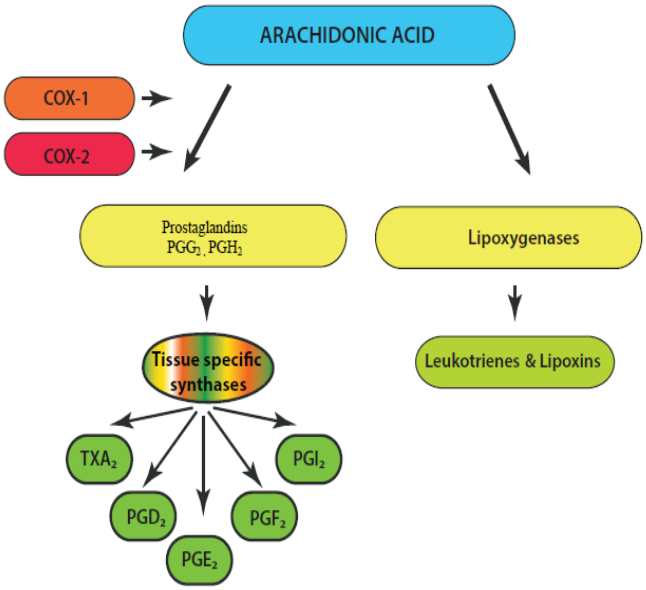

Figure 6. Production of prostaglandins and other eicosanoids. Tissue damage causes arachidonic acid to be split off the cell membrane phospholipids. There are two main pathways; the lipoxygenase pathway leads to the formation of leukotrienes and lipoxins, whereas the COX pathway leads to formation of prostaglandins and thromboxanes.

COX-2 expression is normally absent in the majority of tissues, however it can be induced in response to several intra- or extracellular stimuli, including proinflammatory cytokines, infectious agents, mitogens, hormones, and growth factors[90;92;93]. COX-2 overexpression has also been described in several tumours, including cancers derived from colon[94;95], breast[96;97], urinary bladder[98], lung[99;100], stomach[101] and pancreas[102-108].

The role of COX-2 in carcinogenesis has been investigated most extensively in colonic neoplasia, especially in patients with familial adenomatous polyposis. The results, from most of the studies, indicate a correlation between high COX-2 expression and poor prognosis in colonic cancer[109;110].

5.8.1 COX-2 in pancreatic head adenocarcinomas

Although several studies showed a potential involvement of COX-2 in tumorigenesis and an inverse relationship between COX-2 overexpression and survival rates in malign tumours, results from studies of COX-2 ekspression in tumours located in the pancreatic head are inconsistent.

It is generally acknowledged that COX-2 expression is upregulated in pancreatic cancer cells[102;103;105-108;111;112]. However, how or if COX-2 promotes pancreatic cancer development is still unclear. Many studies show either no relation or positive correlation between COX-2 expression and favourable clinicopathological parameters or survival [103;104;106;107;111-116]. In particular, high differentiation grade was frequently associated with increased COX-2 expression[106;107]. Other publications demonstrate negative effect of COX-2 expression on survival[102;105].

There are also contradictory reports on the use of non-steroidal anti-inflammatory drugs in pancreatic cancer. In some studies, an association between prolonged use of COX-2 inhibitors and increased risk of PC was observed[117;118], while other studies showed the opposite effect[119]. Some other, experimental studies proposed a dual, both stimulating and suppressing effects of COX-2 inhibitors, depending on the degree of COX-2 expression in tumour cells, and the dose of COX-2 inhibitors[120;121].

There are only few publications reporting COX-2 expression in ampullary and distale bile duct carcinoma[122-127]. Although the experimental data on cell lines suggest that COX-2 may play a role in carcinogenesis, angiogenesis or apoptosis[128;129], there is no data indicating an association between COX-2 and survival in these two types of carcinoma. One study reported a shorter median survival in patients with COX-2 positive ampullary carcinomas, however, in this study node metastasis showed no effect on survival, suggesting that the studied patient group might not have been properly selected[127]. The association between COX-2 expression and type of differentiation have been studied in ampullary carcinoma[125] and the results demonstrated a high rate of COX-2 expression in the intestinal subtype (92%). The clinical significance of those results was not established, however, a significant negative correlation was found between T stage and COX-2 expression[125].

5.9 PGE_2

Most cellular effects of COX-2 are performed via its metabolite prostaglandin E_2 (PGE_2). PGE_2 exerts its cellular effect through G-protein- coupled EP and FP receptors [130;131]. The functions of the EP receptors are dictated by the intracellular signalling machinery coupled to each receptor. EP2 and EP4 receptors are G_s-coupled receptors that stimulate adenylyl cyclase activity. This results in an increase of cyclic adenosine monophosphate (cAMP) - the second messenger that has influence on many aspect of cellular function, from differentiation to cell death[132]. EP3 receptors are G_i-coupled receptors that inhibit adenylyl cyclase activity with subsequent decrease in intracellular cAMP. EP1 receptors elevate the intracellular Ca^{2+}-levels through mechanisms that may involve both phospholipase C-dependent and -independent mechanisms [130;131;133], and FP receptors are G_q-coupled

receptors that elevate intracellular Ca^{2+}-levels [130;131]. In addition, several of these receptors may signal via G protein-independent mechanisms [134].

Elevated COX-2 and its metabolite PGE_2 are associated with a wide range of effects, including cellular proliferation, differentiation, angiogenesis and apoptosis[80;84;135-140].

6. Aims of the study

The aims of the present study were to investigate how the standardized histopathological examination protocol can help to correctly classify the tumour origin of pancreatic head adenocarcinomas. Further, we examined how different approaches to assessment of lymph node metastases and expression of COX-2 in the tumours can predict long-term outcome. Finally we assessed COX-2 expression and effects of PGE_2 on cells proliferation and collagen synthesis in pancreatic stellate cells in vitro.

The specific aims of this study were:

1. How does predetermined diagnostic criteria and the experience of the pathologists involved in the histopathological evaluation influence the accuracy of diagnosis of pancreatic head adenocarcinomas?

2. What is the best prognostic factor for long-term survival for pancreatic head adenocarcinomas, N status, lymph node ratio (LNR), or the actual number of metastatic lymph nodes? Do these different measures of lymph node metastasis discriminate differently between prognostic subgroups among the three types of pancreatic head cancers?

3. What is the prevalence of COX-2 expression in tumour tissue of pancreatic head adenocarcinomas, as assessed by immunohistochemistry? Does COX-2 expression have any prognostic relevance? Are there any other prognostic factors associated with COX-2 expression?

4. Which cells in pancreatic cancer tissue express COX-2? Specifically, how is COX-2 expression in the pancreatic stellate cells regulated and what is the role of PGE_2?

7. Summary of results

Paper I

Reclassification of tumour origin in resected periampullary adenocarcinomas reveals underestimation of distal bile duct cancer

Eur J Surg Oncol 2012; 38:1043-1050.

Primary adenocarcinomas removed by pancreatoduodenectomy originate from the duodenum (DC), ampulla (AC), distal bile duct (DBC), or pancreas (PC). Despite the well-established histological definition of the anatomical structures in the specimen, the precise tumour origin may be difficult to determine, especially when the tumour is large and involves more than one anatomical position. Correct diagnosis is essential for appropriate adjuvant therapy, patient inclusion in clinical trials, and the general quality of the clinical data.

In this study, routine histopathology reports of tumour origin performed by multiple pathologists were independently re-evaluated by two experienced pancreatic pathologists, based on predetermined criteria.

We found that slide review changed the diagnosis in 27% of the patients. Distal bile duct cancer was found to be most frequently misdiagnosed. Misclassification of PC was mainly due to erroneous diagnosis of AC. Reassignment of tumour origin caused no significant changes in survival within cancer type, but resulted in a significant difference in survival between DBC and PC.

These results indicate that standardised histopathological evaluation based on predetermined criteria and performed by experienced pathologists can improve accuracy of the diagnosis.

Paper II

Prognostic relevance of number and ratio of metastatic lymph nodes in resected pancreatic, ampullary and distal bile duct carcinomas

Ann Surg Oncol 2013; 20:233-241

In this study we evaluated how N status, lymph nodes ratio (LNR) and number of metastatic lymph nodes predict long- term survival after pancreatectomy for ampullary (AC), biliary (DBC), and pancreatic (PC) adenocarcinomas. We show that lymph node involvement was more frequent in PC than in AC and DBC.

Our results demonstrate that the prognostic value of nodal involvement depends on the cancer origin. In AC and DBC, N status clearly discriminates between subgroups of patients with different long-

term survival, whereas number of metastatic nodes and LNR do not predict survival among node-positive resections. In PC, N status does not discriminate between prognostic groups. However, increasing LNR is significantly associated with poorer survival both in unadjusted analysis, as well as when adjusting for margin involvement, degree of differentiation, and tumour diameter. In addition, the data demonstrated that LNR is more predictive of long- term outcome when the number of lymph node assessed is insufficient.

Paper III

COX-2 overexpression in resected pancreatic head adenocarcinomas correlates with favorable prognosis

BMC Cancer 2014; 14:458

Overexpression of cyclooxygenase-2 (COX-2) has been implicated in oncogenesis and progression of adenocarcinomas of the pancreatic head. The data on the prognostic importance of COX expression in these tumours is inconsistent and conflicting. In this paper we evaluated how COX-2 overexpression affected overall postoperative survival in pancreatic head adenocarcinomas. COX-2 immunohistochemistry was performed on whole tumour slice.

Our results demonstrate that COX-2 is overexpressed in pancreatic cancer, ampullary cancer and distal bile duct cancer and confers a survival benefit in all three cancer types. The overexpression is consistently linked to the histopathological type of differentiation and to the degree of differentiation. Moreover, in pancreatic cancer, COX-2 independently predicts a favourable prognosis.

Paper IV

Inhibitory effects of prostaglandin E2 on collagen synthesis and cell proliferation in human stellate cells from pancreatic head adenocarcinoma.

BMC Cancer 2014; 14:413

Several studies have described an increased cyclooxygenase-2 (COX-2) expression in pancreatic cancer, but the role of COX-2 in tumour development and progression is not clear. The aim of the present study was to examine expression of COX-2 in cancer cells and stromal cells in pancreatic cancer specimens, and to explore the role of PGE_2 in pancreatic stellate cell proliferation and collagen synthesis.

We performed immunohistochemistry and immunofluorescence on slides from whole sections of tissue blocks using antibodies against COX-2 and α-smooth muscle actin (αSMA). Pancreatic stellate cells (PSC) were isolated from surgically resected tumour tissue by the outgrowth method.
Our results show that COX-2 is present in pancreatic carcinoma cells, but not in stromal cells. Cultured PSC however express COX-2, which can be further induced by interleukin-1β (IL-1β), epidermal growth factor (EGF), thrombin, and PGE_2, but not by transforming growth factor-β1 (TGFβ). Indirect coculture with the adenocarcinoma cell line BxPC-3, but not HPAFII or Panc-1, induced COX-2 expression in PSC.
We further showed that treatment of PSC with PGE_2 strongly stimulates cAMP accumulation, mediated by EP2 receptors, and also stimulates phosphorylation of extracellular signal-regulated kinase (ERK). Treatment of PSC with PGE_2 or forskolin suppresses both TGFβ-stimulated collagen synthesis and PDGF-stimulated DNA synthesis.
Our results demonstrate that COX-2 is mainly produced in carcinoma cells and suggest that the cancer cells are the main source of PGE_2 in pancreatic tumours. PGE_2 exerts a suppressive effect on proliferation and fibrogenesis in pancreatic stellate cells. These effects of PGE_2 are mediated by the cAMP pathway and suggest a role of EP2 receptors.

8. Discussion

8.1 Methodological consideration

8.1.1 Patient selection

The patient groups included in the present study comprised individuals who underwent a standard pancreatoduodenectomy (Whipple's procedure) for pancreatic head adenocarcinoma between 1998 and 2011 at Oslo University Hospital, Rikshospitalet.

The study protocol and patient consent documents were approved by the Regional Committee for Medical and Health Research Ethics, and was in compliance with the Helsinki Declaration. The study included only adults. Since all Norwegian inhabitants receive a unique personal identification number, it is possible to trace information about death date for nearly all citizens. In the presented study, no patients were lost to follow-up. For survival studies, patients were followed until death or censored after maximum five years (60 months).

Hospital records were reviewed retrospectively, and standard demographic, clinicopathological, and tumour-specific data were collected.

Patients included in the study were operated with curative intent for primary adenocarcinomas in the pancreatic head, including ampullary adenocarcinoma (AC), distal bile duct adenocarcinoma (DBC) and pancreatic adenocarcinoma (PC).

Paper I and II included 179 patients operated between 1998 and 2009. For the purpose of data presented in the paper I, 28 patients with diagnosis of the adenocarcinoma of the periampullary duodenum (DC) operated during the same period, were also included.

In paper III, patients with diagnosis AC, DBC or PC operated during 2010-2011 were also included giving a total of 230 patients studied..

Overall in this thesis, 261 consecutive pancreatoduodenectomies for PC 92 (35%), AC 62 (24%), DBC 76 (29%) or DC 31 (12%) operated during 1998-2011 were studied.

8.1.2 Histopathological assessment

Most of the resection specimens (230) were routinely handled with the bisectional dissection method[141] and examined according to a standardised protocol as described by Westgaard at al. [142]. After introduction of the axial slicing method[143] in 2010, 31 consecutive pancreatoduodenectomy specimens were handled according to this new technique.

The following histopathologic factors were prospectively registered in routine practice: tumor origin, type of differentiation, maximum tumour diameter, degree of differentiation, perineural infiltration, vascular infiltration, ductal dysplasia, lymph node status, and resection margins involvement. For identification of the anatomical origin of the tumour, blocks were made by sectioning parallel to these structures and including the duodenum and ampulla of Vater, in order to demonstrate the relation of the tumour to these structures. As from 2003, in the majority of cases whole mount blocks were made for identifying the relation of the tumours to the critical structures. If necessary, further cross sections of the tumour were made to evaluate tumour size and infiltration into adjacent structures.

The primary histopathological evaluation was performed by different pathologists involved in the routine evaluation. As many of them were not attached permanently to the pancreatic unit at the Department of Pathology, the number of examinations of the present material performed by a single pathologist varied between two and 68. The four pathologists with the highest workload together assessed 87% of the total cohort. In the routine assessment of tumour origin pathologists followed generally established guidelines. However, due to different level of experience of the pathologists involved, the quality and accuracy of the assessment varied among assessed cases.

8.1.2.1 Tumour origin

In all cases retrospective reevaluation of the cancer origin was performed.
All registered parameters of the prospectively collected database, including anatomic site of tumour origin, were independently reevaluated by slide review performed by an experienced gastrointestinal pathologist (OPFC). In paper I, due to the study design, additional reevaluations were also performed by another experienced pathologists (KG). Both pathologists were also involved in the primary, routine assessment of the specimens.
During reevaluation of tumour origin, the two pathologists followed the general guidelines also used under routine evaluation. The main criteria included estimation of the centre of the tumour mass, tumour location relative to ductal, duodenal and pancreatic anatomy, and the presence of epithelial dysplasia or *in situ* neoplasia. In large tumours, cancer infiltration located at both sides of the bile duct and /or presence of BilIN were generally considered to indicate bile duct origin. Presence of PanIN in the pancreatic duct or dysplasia in the ampulla suggested pancreatic or ampullary origin, respectively. Extension and location of the central tumour mass also played an important role in the final evaluation of the site of tumour origin. Many duodenal/papillary tumours showed extension into the ampulla, and, conversely, ampullary tumours could affect the duodenum, rendering many of these

tumours difficult to classify. In these cases extension of *in situ* alterations and location of the central tumour mass were taken into account.
Following the independent reassessment by the two expert pathologists, cases that had been assigned to different locations were identified, and subjected to further discussion for obtaining a consensus decision, which thus constituted the final classification. All tumours were allocated to one of the following locations: duodenum, ampulla, distal bile duct or pancreas.
Upon slide review, the two pathologists were blinded to the original diagnosis. The clinical and macroscopic data were available, as had been the case during the primary evaluation.

8.1.2.2 Lymph node assessment

Pancreatoduodenectomy without radical lymph node resection was performed in our hospital as a standard procedure. Lymph nodes were obtained by *en bloc* dissection of primary metastatic regions, not including secondary sites such as the peri-aortic lymph nodes[39-42] (Figure 3).
Nodal status was registered prospectively by routine pathologists according to the standardized protocol. Numbers of positive and total lymph nodes were extracted from the pathology reports, and LNR was calculated according to these numbers. The present study did not discriminate between nodal involvement due to direct invasion or true metastasis, in accordance with recommended principles for staging of nodal involvement[144].

8.1.3 Immunohistochemistry

In Paper III, COX-2 expression in the tumours of the pancreatic head was evaluated by immunohistochemistry. Monoclonal COX-2 antibodies were applied to 230 whole tumour sections. Prior to the final immunostaining, multiple testing of buffer type and antigen dilutions was performed. The optimal antibody dilution was established as 1:25.
For each batch of tumour sections to be stained, positive and negative control were determined. Islets of Langerhans and/or duodenal mucosa were moderately to strongly positive for COX-2, also in those tumours with no COX-2 expression, and served as internal positive controls. Additionally, sections with omission of the primary antibody were used as negative controls.
To test the validity of the Thermo antibody used for the study cohort, we performed additional immunostaining with a different monoclonal COX-2 mouse antibody, Invitrogen (Camarillo, CA, USA), on duplicates of twenty pancreatic cancer slides from the study cohort. The results of Thermo and Invitrogen immunohistochemistry were identical (Paper III, Fig.1e). As the Thermo antibody was

not suitable for western blotting (producer recommendation), further validations of the immunochemistry by western blotting was performed only with the Invitrogen antibody. The results showed a highly specific band for COX-2 (Paper III, Fig.1f).

8.1.3.1 Evaluation of COX-2 immunostaining

Since COX-2 expression in pancreatic tumours often is heterogeneous [104;111;145], the actual number of COX-2 positive tumours might easily be underestimated if tissue microarrays are used. In our study immunohistochemistry was therefore performed on whole slide sections and assessed on multiple high-power fields within each tumour.

The COX-2 expression was evaluated by the intensity and the extension of staining. Tumours with moderate and strong staining were considered positive while tumours with no or faint staining were regarded as negative. COX-2 staining showed considerable heterogeneity within the tumours. Thus, the final immunoscore of COX-2 expression was calculated as an average of five different extension scores in the tumours considered positive on the intensity evaluation.

To ascertain the reliability of the interpretation of the immunostaining, almost half of study specimens (44%) were tested for the interobserver variability with respect to scoring of the stained tumours. A kappa-value of 0.73 (substantial agreement) was found.

8.1.4 Isolation of pancreatic stellate cells

One of the characteristic histological feature of pancreatic cancer is the prominent fibrosis surrounding the cancer cells. There is increasing evidence that pancreatic stellate cells are playing a critical role in this extensive stromal reaction known as tumour desmoplasia. To study the role of pancreatic stellate cells and particularly the effects of COX-2 and PGE_2 on their proliferation and collagen production, we isolated and cultured pancreatic stellate cells from patients with resectable pancreatic head adenocarcinoma.

Small tumour samples for isolation of the human pancreatic stellate cells were obtained during pancreatic surgery from patients operated in the period 2009 - 2011. All samples were coded to protect the identities of the subject participating in this study. The study protocol and patients consent documents were approved by the Regional Committee for Medical and Health Research Ethics, and complied with the Helsinki Declaration.

8.1.4.1 Cell isolation and culture

Two main methods have been developed in recent years for isolation of pancreatic stellate cells. The isolation of quiescent PSCs from rat pancreas, using a centrifugation method, was first developed by Apte et al[73]. This method was based on the density gradient caused by the presence of the lipid content in the stellate cells in their quiescent state.

However, this method was not suitable for isolation of human pancreatic stellate cells from resection specimens from patients operated for pancreatic adenocarcinoma. Firstly, the available amount of tissue was strictly limited, both by the tumour size itself, and by demands from pathologist for routine diagnostics. Only when the pathologist had secured what was considered a sufficient amount of tissue for histological diagnosis, surplus tissue could be excised from the specimen for research purposes. As the amount of tissue might be very sparse, the centrifugation method of Apte at al[73] was not easy to perform. The other main problem was the activation state of the stellate cells. As stellate cells become activated, which is the case during changes in the pancreas caused by cancer, they were losing lipid droplets, and the centrifugation method might fail. The last problem was the vast fibrotic reaction in the tissue form the pancreatic adenocarcinoma, which made it difficult to digest it properly prior to the centrifugation[146].

Bachem et al.[74] described a different method for isolation of pre-activated human PSCs from resected tissue. This so-called outgrowth method of Bachem was used in our laboratory for the present study. Briefly, pancreatic tissue blocks (100–150 mg) were obtained during pancreatic surgery from patients with resectable pancreatic head adenocarcinoma. The tissue blocks were cut using a razor blade (0.5–1 mm^3) and seeded in uncoated culture wells. Cells were cultured at 37˚C in 5% CO_2 / air humidified atmosphere. After reaching confluence, monolayers were trypsinized and passaged 1:3. The purity of the cells was assessed by morphology (most cells were stellate-like, with long cytoplasmatic extensions; some were also spindle shaped) and cytofilament staining of α-SMA and Vimentin (Fig.7).

All experiments were performed using cell populations between passage 4 and 8.

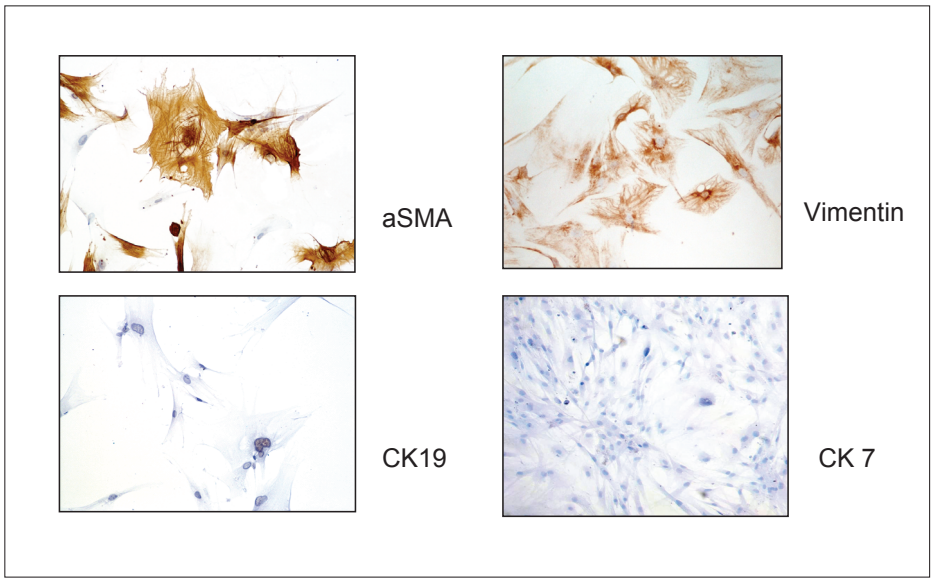

Figure 7. Cultured pancreatic stellate cells. Cells are stellate-like, with long cytoplasmatic extension and have cytofilament staining of α-SMA and Vimentin. None of the cells are positive for cytokeratins 7 or 19 characteristic for ductal adenocarcinomas

The cultured pancreatic stellate cells were assessed after different passages with immunofluorescence staining with αSMA and COX-2. (Fig.8)

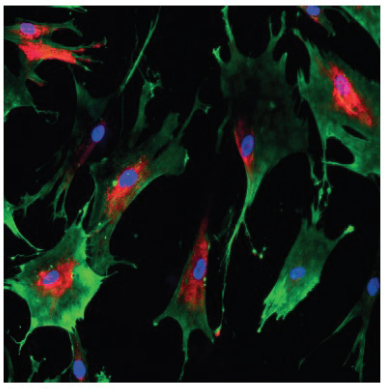

Figure 8. Immunofluorescence staining of cultured pancreatic stellate cells, passage five; COX-2 positive cells - red colour, αSMA positive cells - green colour, nucleus - blue colour.

8.2 Discussion of results

In this thesis, we assessed the accuracy of the histopathological assignment of exact anatomical site of origin in the tumours of the pancreatic head (Paper I). Furthermore, we studied standard prognostics factors and proposed new variables as the predictors for long-term survival for patient operated with pancreatoduodenectomy for adenocarcinomas in this region. The included papers (Paper II and III) demonstrate particularly the impact of lymph node status, LNR and COX-2 expression on prognosis. Lastly, in Paper IV, we studied PGE_2 effects on pancreatic stellate cells.

8.2.1 Tumour Origin

Adjuvant treatment strategies and long-term survival differ considerably among the different types of adenocarcinomas in the pancreatic head. Failure to obtain a correct diagnosis leads to negative effects for the individual patient as the organ of origin determines inclusion in current postoperative adjuvant therapy regimens. For clinical trials on adjuvant treatment where tumour origin is the essential inclusion criterion, incorrect diagnosis may lead to false assumptions regarding the effectiveness of the therapy being given.

Although the importance of the correct diagnosis is widely accepted, several lines of evidence suggest that the distinction between pancreatic, ampullary, distal bile duct and duodenal cancer, in series of pancreatoduodenectomies, might not always be reported in an accurate manner. Firstly, a reported relative incidence of the four cancer types in the several large European and American centres show large wide variation, which can not be explained solely by patient selection, and might cast doubts on the diagnosis precision[5;6;34-37;39;143;147;148]. Furthermore, several studies on the reported long – term survival after pancreatoduodenectomy directly question the accuracy and precision of the histopathological diagnosis[149-151]. Relatively high frequency of PC diagnosis combined with comparatively low frequency of the AC and DBC diagnoses in many series, might indicate that PC can be a default diagnosis, especially when histological assessment is particularly difficult[5;6;34-37;39;143;147;148]. This results in extensive variation in the incidence of PC diagnosis, which varies from 38% to 76% in different series of pancreatoduodenectomies [5;6;34-37;39;143;147;148].

In this thesis, for the first time in a structured manner, we critically assessed the routine classification of tumour origin and investigated different aspects of the histopathological evaluation (Paper 1). The study demonstrates that slide review performed by experienced pancreatic pathologists with particular emphasis on determining the tumour origin based on predefined criteria is necessary for

correct assignment of tumour origin. The experience of the pathologists, and their attitude and efforts spent to determine the true tumour origin, was found to be more important than particular differences in specimen dissection, like the number of tissue block examined from each tumour or whether whole mount blocks were made or not.

The most experienced pathologists, with the highest numbers of histopathological examinations, generally had fewer reclassified cases compared to the less experienced pathologists. This is probably a general phenomenon and not only limited to pathologists in Norway. A survey performed in the United Kingdom showed that only 20% of the pathologists actively distinguish DBC from PC, 13% reported that thy did not do so usually, while the remaining 67% reported that they just tried to make distinction[152;153].

As histopathological assessment of tumour origin in pancreatoduodenectomies is demanding, it should be noted that generally accepted international consensus guidelines regarding recommended slicing techniques and assessment of tumour sections are still lacking[152;153].

The recently introduced technique of axial slicing has generally been shown to be superior to the bisectional technique[143].

After the completion of Paper I, we have introduced this method in our institution and the 31 consecutive pancreatoduodenectomies were handled according to this technique.

The conclusions of the study in Paper I and the introduction of method of axial slicing have increased the precision in establishing correct diagnosis in our department. At the end of the study period of 261 specimens obtained during pancreatoduodenectomy, 230 specimens were handled with the bisectional technique, and 31 with axial slicing (Table 3). In the DBC group, the accuracy of the diagnosis of the tumour origin increased from 52% to 73%, in the PC group from 78% to 93%, and in the DC group from 87% to 100%. In the AC group the site of origin reminded generally unchanged (84 - 80%) presumably due to the small number of patients (5) where specimens were handled with the axial slicing method.

Table 3.

Accuracy of the diagnosis		
	Bisectional slicing technique (230 patients, 88%)	**Axial slicing technique (31 patients, 12%)**
DBC	52 %	73 %
PC	78 %	93 %
DC	87 %	100 %
AC	84 %	80 %

Although the number of cases assessed with the new technique was relative low 31 (12%), the results clearly showed that the combination of a new, better slicing technique with particular focus on correct tumour diagnosis among pathologists, could improve the quality of the histopathological evaluation.

Taken together the study indicates that quality of the histopathological diagnostic work on pancreatic head tumours benefits from keeping this to experienced pancreatic pathologists in high- volume centres, just as what is now generally accepted for the surgical treatment of these tumours [154]

8.2.2 Prognostic factors in pancreatic head adenocarcinomas

Since PC, AC, DBC and DC are separate cancer entities, different pathological variables may have different prognostic significance in these cancers. In paper II and III we assessed how lymph node status, lymph node ratio (LNR), and COX-2 expression may predict long-term survival in PC, DBC and AC. Tumours originated in the duodenum (DC), although removed by the same surgical procedure, are biologically and molecularly more related to tumours originated from the intestine (small bowel and colon) and were therefore excluded from this analysis.

8.2.2.1 Evaluation of the nodal tumour involvement

In the studies presented in paper II, we aimed to ascertain whether N status, number of metastatic lymph nodes or lymph node ratio (LNR) better predicts long- term survival after pancreatoduodenectomy for PC, DBC and AC. Previous studies that have approached this question have mostly been based on nonstandardized evaluation of the cancer origin[3;143;150;154-156]. In our studies, all results were based on tumour classification where the anatomic site of tumour origin had been subjected to reevaluation by slide review. This might at least partly explain the conflicting data as to which method is the better for the evaluation of nodal metastasis in adenocarcinomas arising from the separate anatomic locations (Table 2, Paper II).

In the AC and DBC groups, lymph node involvement was significantly less frequent (48% and 57%, respectively) than in the PC group, where more than 75% of the patients had lymph node metastasis. In AC and DBC, nodal status distinctly predicted long- term survival, while LNR (among N1 resections), was insufficient as a prognostic indicator.

In the group of PC, there was no difference in overall survival between n N0 and N1 resections. This may be explained by the observation that there is no difference in long-term prognosis between patients with involvement of 0 or 1 lymph node (Paper II, Fig.2b). It is known that in some cases, involvement of one lymph node may be a consequence of direct lymph node invasion and thus not

representing true metastasis. The overall survival for these patients is similar compared to overall survival for patients with node - free resection[157]. However, in the current TMN system recommendation, all these patients are classified as N1[158]. The other possible explanation for the similar long-term survival of patients with no or one lymph node metastasis could be attributed to the aggressive biology of PC itself. The fact that the tumour originates from the pancreatic tissue is more biologically important than the presence of none or one lymph node metastasis[3].

In our study we provided evidence that in patients with pancreatic cancer diagnosis, LNR significantly discriminated between patients with good and poor prognosis, in the whole cohort of patients (N0 and N1 resections), and in patients with N1 resections alone.

The optimal cut-off value of LNR is defined as the value that best stratifies patients with respect to cancer-specific survival. In our study, the LNR cut-point was defined as 0.2. In studies on LNR the cut-point used to determine patients group with different prognosis, has been between 0.15 and 0.20[159;160]. A study of House[161], specially designed to calculate the most optimal LNR cut-off, concluded that it might be 0.18. However, the mean number of evaluated lymph nodes in that study was 17, which is slightly higher than what is found in most other studies. The cut-off point of LNR obviously depends on the average number of evaluated nodes[159-161;161]. In our study, the median number of lymph nodes was eleven. The number was lower compared to the recommendation by AJCC[162] (twelve nodes), however it was within the last recommendation from UICC (ten nodes)[163]. The median number of evaluated lymph nodes is most frequently ≤ 9 even in academic institutions[164], A high lymph node yield could also in some reports result from resections involving extended lymphadenectomy[165]. The median number of lymph nodes evaluated in the present study is thus representative of a medium-volume tertiary referral institution.

Taken together our results indicated that N status evaluation was sufficient and superior to LNR in nodal evaluation for AC and DBC. Those results were confirmed in multivariate analysis, where N status was an independent prognostic variable in AC and DBC.

In PC, in contrast, N status was insufficient as a prognostic factor. Furthermore, in PC LNR was significantly associated with overall survival and thus serves as an independent predictor of long-term prognosis.

Importantly, the finding that LNR is a better predictor of survival than node status or tumour size in PC raises the question whether standard TNM classification in pancreatic tumours could be extended to include LNR data. Since our study is limited by a relatively low number of patients, larger studies, where anatomic tumour origins are properly examined/determined, are needed to confirm these results.

8.2.2.2 COX-2 expression

In Paper III, we assessed by immunohistochemistry, whether COX-2 expression predicts long-term survival in resected PC, DBC and AC. Our study is the first which compares COX-2 expression levels in these three types of cancer in a setting where the tumour origin has been determined by standardised histopathological evaluation.

We found that COX-2 expression was very similar in all three tumour types, with positivity rate over 70%. Overall patient survival was more favorable in COX-2 positive than COX-2 negative tumours in all tumour types (Paper III Fig 2 a-c). This was particularly prominent in AC and PC. In DBC the same trend was observed, however, not reaching significance.

As the precise function of COX-2 in development of tumours in the pancreatic head is not fully understood, our results may contribute to elucidating some features of the tumours, including the association between histologic differentiation and COX-2 expression.

Previous studies have shown that the histologic *type of differentiation* of tumours of the pancreatic head significantly influences patients' survival[155;166]. Tumours with intestinal type of differentiation have generally a more favorable prognosis than tumour with pancreaticobiliar type[155;166]. When exploring the potential relationship between differentiation type and COX-2 expression, we found that all of the PC and DBC tumours, and over 80% of the AC with intestinal type of differentiation were COX-2 positive. The survival data of intestinal tumours in AC showed a particularly favorable prognosis for patients with tumours expressing COX-2. Taken together this confirms that COX-2 expression in the tumours of the pancreatic head is closely related to the intestinal type of differentiation. As the survival of the patients with tumours exhibiting intestinal type and COX-2 positivity are particularly beneficial, these two variables might be important factors for patients' prognosis and hence useful in future trials for stratification of patients in adjuvant therapy.

A different mechanism of COX-2 influence on survival may be the possible association with the *degree of differentiation* of the tumours. In paper III we assessed COX-2 expression in relation to commonly studied clinicopathological variables. We focused on the largest group in our study, PC, and did not find any association between COX-2 expression and lymph node status, LNR, R-status, tumour diameter, vascular or perineural infiltration. We found however that COX-2 positivity in tumours was strongly associated with high degree of tumour differentiation (Grade I and II). Our result are in agreement with studies of cultured pancreatic cancer cells, where only moderately and highly differentiated pancreatic cancer cell lines expressed COX-2 [107;112;167], and with studies on human pancreatic adenocarcinoma tissue, where high COX-2 expression was observed mainly in well differentiated lesions[145]. As we expected, the joint effects of COX-2 status and differentiation

grade on survival showed that the subgroup of patients with COX-2 positive/well differentiated tumours had a significantly better survival compared to patients with COX-2 negative/poorly differentiated tumours. Thus the presence of COX-2 expression in these tumours appears to be a marker of favorable prognosis closely linked to the degree of tumour differentiation.

In Paper II we showed that LNR independently predicted survival in resected pancreatic cancer. To explore this further, we examined also the joint effects of COX-2 status and LNR. The results clearly showed that patients with COX-2 negative tumours and unfavorable LNR (LNR >0.2) had the worst prognosis (Paper III, Fig. 3b). Finally, multivariate analysis showed that COX-2 negativity of the tumours and high LNR (LNR>0.2) independently predicted poor prognosis (Paper III, Table 2). The grade of differentiation could not be included in the same analysis as this was strongly correlated to COX-2.

Our results are partly contrary to two large published studies which, found that COX-2 positive tumours were associated with reduced survival in patients with pancreatic cancer [102;105]. Although the patients groups in these studies are larger than in our study, the methodological differences in the selection of patients and / or immunohistochemical techniques employed, raise some doubts on the results of these studies. In the study of Juuti[102], more than 30 years old specimens were included. It is well known that for immunohistochemical staining protocol, aging of the fixed tumour tissue might interfere with staining process[168]. Moreover, the variations in fixation protocols over time might also result in inadequate staining. In the study of Matsubayashi[105], COX-2 staining was performed on tissue microarrays. Since COX-2 expression is often heterogeneous[104;111;145] in the pancreatic cancer, the actual number of COX-2 positive tumours in this study might be underestimated.

Taken together, the results from Papers II and III demonstrated that both LNR and COX-2 expression are independent prognostic factors in pancreatic cancer and might therefore be used for stratification of patients for clinical trials with adjuvant treatment.

8.2.3 PGE_2 effects in pancreatic cancer stroma

In Paper IV we studied the involvement of the major product of COX-2, PGE_2, in the interaction of the pancreatic stellate cells with cancer cells. To examine the cellular mechanism of these relationships, we studied cultured pancreatic stellate cells, and explored the effect of PGE_2 at a cellular level.

Immunohistochemistry performed on tumour sections from patients with PC, showed COX-2 expression in the cancer cells and strong staining with α-SMA, an indicator of the presence of activated stellate cells, in the tumour stroma. However, we did not find any detectable double staining

of COX-2 and α-SMA in the stroma. This was further examined by immunofluorescence, with the same outcome. However, in the cultured stellate cells, COX-2 expression was clearly detected, and was constant in different passages.
Co-culture of pancreatic cancer cells with PSC resulted in induction of COX-2 in the stellate cells, and showed that this induction apparent to be mediated by interleukin I, secreted from the cancer cells. We also found that PGE_2, via cAMP, inhibited collagen- and DNA-synthesis in the stellate cells.

Multiple lines of evidence suggest that pancreatic stellate cells (PSC) have an essential role in the development of pancreatic cancer desmoplasia [10;70;75;85;146]. Studies of human and rat PSC in culture have identified a number of growth factors, cytokines, and hormones as regulators of pancreatic stellate cell activation [70]. However, PGE_2 influence on the function of the pancreatic stellate cells, particularly the effect on pancreatic fibrosis and cell proliferation, is still uncertain.
To gain insight into the mechanism of PGE_2 effect in pancreatic stellate cells, we first examined PGE_2 signalling pathways in these cells. PGE_2 may affect cells through EP and FP receptors[130;131]. Pancreatic stellate cells express mainly EP2 and EP4 receptors[80;81;169] with cAMP as a second messenger.
We therefore examined the effect of PGE_2 on cAMP accumulation in the stellate cells and found that PGE_2 induces strong, dose-dependent accumulation of cAMP (Paper IV, Fig.3). We also found that PGE_2 induced cAMP accumulation mainly through stimulation of EP2 receptors. Inhibition of the EP4 receptor did not show any changes in cAMP accumulation.
Furthermore, we tested the effect of PGE_2 on collagen synthesis. It is known that TGFβ1 stimulates collagen synthesis in pancreatic stellate cells[70;76;77]. In our study, we could not detect any direct effect of PGE_2 on collagen synthesis. However, we observed that treatment with PGE_2 suppressed the increase in collagen synthesis stimulated by TGFβ1. As the same effect was observed by the treatment with forskolin (a direct activator of adenylyl cyclase), the result suggests that it was a cAMP mediated effect. As we could not observe induction of COX-2 in the stellate cells by TGFβ1, pretreatment with indomethacin (non-selective COX inhibitor) did not affect TGFβ1-induced collagen synthesis.
We then examined how PGE_2 affects stellate cell proliferation. In our experiments, we could not detect any direct effect of PGE_2 or forskolin on DNA synthesis. However, both PGE_2 and forskolin significantly inhibited PDGF- stimulated DNA synthesis, suggesting that this effect was mediated by cAMP. Pretreatment of the cells with indomethacin did not affect PDGF- stimulated DNA synthesis.

Taken together we found that COX-2 is mainly expressed in carcinoma cells, suggesting that the cancer cells are the main source of PGE_2 in pancreatic tumours. In the pancreatic stellate cells, PGE_2 has both the antiproliferative and antifibrotic effects (Paper IV). Thus, inhibition of COX-2 may inadvertently accelerate fibrosis progression in pancreatic cancer.

9. Conclusions

Addressing the specific aims of our studies, the main conclusions of this thesis are the following:

1. Histopathological evaluation and exact diagnostic identification of tumour origin in the patients operated with pancreatoduodenectomy is challenging. We found that slide review changed the diagnosis in 27% of the patients. Distal bile duct cancer was found to be most frequently misdiagnosed (53%). Precise predetermined diagnostic criteria, with special focus on the anatomical site of origin, are essential to improve the accuracy of diagnosis. Furthermore, high workload per pathologist increases the precision of the histopathologic diagnosis.

2. In patients operated with pancreatoduodenectomy, N status and LNR are superior to the number of metastatic nodes as prognostics indicators. The predictive value of these variables depends on the cancer origin. In ampullary and distal bile duct cancer, N status discriminates between subgroups of patients with different long-term survival whereas in pancreatic cancer, LNR is clearly more powerful in prognostic subclassification. In patients with pancreatic cancer, multivariat analysis identified LNR > 0.2 as an independent predictor of poor long-term survival. LNR could therefore be proposed as a standard, alternative measure of nodal involvement in pancreatic cancer.

3. COX-2 is overexpressed in more than 70% of pancreatic, ampullary and distal bile duct cancers and is associated with the histopathological type of differentiation, with the degree of differentiation, and with a favourable prognosis. In pancreatic cancer, in a multivariate model, COX-2 negative tumours and LNR > 0.2, independently predicted poor prognosis

4. When assessed by immunohistochemistry, COX-2 is mainly expressed in pancreatic carcinoma cells, and these cells are regarded as the main source of PGE_2 in pancreatic cancer tumour tissue. COX-2 was not detected in the stroma, however COX-2 was detected in the cultured pancreatic stellate cells (PSC), and could be further induced by interleukin-1β (IL-1β), epidermal growth factor (EGF), thrombin, and PGE_2, but not by transforming growth factor-β1 (TGFβ). Treatment of PSC with PGE_2 suppressed both TGFβ-stimulated collagen synthesis and PDGF-stimulated DNA synthesis, suggesting that inhibition of COX-2 may inadvertently accelerate fibrosis progression in pancreatic cancer.

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RESEARCH ARTICLE Open Access

COX-2 overexpression in resected pancreatic head adenocarcinomas correlates with favourable prognosis

Ewa Pomianowska[1,2*], Aasa R Schjølberg[1,3], Ole Petter F Clausen[3] and Ivar P Gladhaug[1,2]

Abstract

Background: Overexpression of cyclooxygenase-2 (COX-2) has been implicated in oncogenesis and progression of adenocarcinomas of the pancreatic head. The data on the prognostic importance of COX expression in these tumours is inconsistent and conflicting. We evaluated how COX-2 overexpression affected overall postoperative survival in pancreatic head adenocarcinomas.

Methods: The study included 230 consecutive pancreatoduodenectomies for pancreatic cancer (PC, n = 92), ampullary cancer (AC, n = 62) and distal bile duct cancer (DBC, n = 76). COX-2 expression was assessed by immunohistochemistry. Associations between COX-2 expression and histopathologic variables including degree of differentiation, histopathologic type of differentiation (pancreatobiliary vs. intestinal) and lymph node ratio (LNR) were evaluated. Unadjusted and adjusted survival analysis was performed.

Results: COX-2 staining was positive in 71% of PC, 77% in AC and 72% in DBC. Irrespective of tumour origin, overall patient survival was more favourable in patients with COX-2 positive tumours than COX-2 negative ($p = 0.043$ in PC, $p = 0.011$ in AC, $p = 0.06$ in DBC). In tumours of pancreatobiliary type of histopathological differentiation, COX-2 expression did not significantly affect overall patient survival. In AC with intestinal differentiation COX-2 expression significantly predicted favourable survival ($p = 0.003$). In PC, COX-2 expression was significantly associated with high degree of differentiation ($p = 0.002$). COX-2 and LNR independently predicted good prognosis in a multivariate model.

Conclusions: COX-2 is overexpressed in pancreatic cancer, ampullary cancer and distal bile duct cancer and confers a survival benefit in all three cancer types. In pancreatic cancer, COX-2 overexpression is significantly associated with the degree of differentiation and independently predicts a favourable prognosis.

Background

Primary adenocarcinomas located in the pancreatic head arise from the ampulla, the distal bile duct, or the pancreatic ductal structures. Due to the topological proximity of these structures, resectable adenocarcinomas arising from any of these three anatomical locations are typically resected by the same surgical procedure, i.e. curative-intent pancreatoduodenectomy. The considerable variation in reported frequencies for the individual tumour sites suggests that the precise tumour origin may be difficult to determine [1] and that the applied methods for histopathological determination of the cancer origin varies widely among institutions [2]. Adenocarcinomas from all three locations may be of pancreatobiliary or intestinal type of differentiation [3].

Overexpression of cyclooxygenase-2 (COX-2) has been described in several tumours, including colon, stomach, breast, lung, and urinary bladder [4-16]. The COX-2 expression is a component of the cellular response to inflammation and is induced by several extracellular or intracellular stimuli, including proinflammatory cytokines, infectious agents, mitogens, hormones and growth factors [17,18]. Several studies have reported overexpression of COX-2 in subsets of pancreatic adenocarcinomas

* Correspondence: ewa.pomianowska@medisin.uio.no
[1]Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway
[2]Department of Hepato-pancreato-biliary Surgery, Oslo University Hospital, Rikshospitalet, PO Box 4950, Nydalen, 0424 Oslo, Norway
Full list of author information is available at the end of the article

in 37 – 80% of the tumours investigated [19-26]. Increased COX-2 expression has also been demonstrated in pancreatic intraepithelial neoplasias (PanINs) [27-30]. However there is relatively few data on COX-2 expression in the two other types of pancreatic head adenocarcinomas, ampullary cancer [31-33] and distal bile duct cancer [34]. Data on prognostic relevance of COX-2 overexpression in all these tumours has been inconsistent and conflicting although most reports indicate an inverse relationship between COX-2 overexpression and survival rates in pancreatic cancer [19,21] and ampullary cancer [32].

The aim of the present study was to examine the prognostic relevance of COX-2 expression in adenocarcinomas from the three separate anatomical sites of origin in the pancreatic head. The data shows that COX-2 is overexpressed in all three types of pancreatic head adenocarcinomas and that COX-2 overexpression is associated with better survival. In contrast to previous reports, COX-2 overexpression was found to be an independent prognostic factor for better survival in pancreatic adenocarcinoma.

Methods

Patients

The study included 230 consecutive patients (103 women and 127 men) undergoing a standard Whipple's procedure for adenocarcinoma with curative intent 1998 -2011 at Oslo University Hospital, Rikshospitalet. The study was approved by the Regional Committee for Medical and Health Research Ethical for Southern Norway.

Standard demographic, clinicopathological, and tumour-specific data were collected retrospectively from hospital records. Overall survival data was obtained from the Norwegian Population Registry, updated June 20, 2013. Since all Norwegian inhabitants receive a unique personal identification number, no patients were lost to follow-up in the present study. Patients were followed until death or censored after maximum five years (60 months). By the end of the study 177 patients were dead. Median follow-up for the remaining 53 patients was 62 months (interquartile range 29 -119 months). Perioperative death (defined as death within 30 days of operation) was included in the analyses (four patients). Analysis excluding perioperative death gave similar results. None of the patients received preoperative chemotherapy or chemoradiotherapy. From 2008, adjuvant chemotherapy with 5-fluororuracil was recommended for eligible patients operated for pancreatic cancer. Thirty-nine percent of the patients (13 of 33) operated in this period received adjuvant chemotherapy (5-FU-based in 11 patients, 2 patients received gemcitabine).

Histopathological evaluation of resection specimens

The resection specimens were examined according to a standardized protocol as described previously [1,35]. All registered parameters of the prospectively collected data base, including anatomic site of tumour origin, where later reevaluated by slide review [1]. The histological type of differentiation was evaluated and all tumours were classified either as intestinal or pancreatobiliary type [3,36]. In brief, pancreatobiliary tumours typically have simple or branching glands and small solid nests of cells surrounded by a desmoplastic stroma, and have cuboideal to low columnar epithelium arranged in a single layer and the nuclei are rounded but with marked variation in size and shape from one cell to the next. Intestinal tumours typically resembled colon cancer, have tall and often pseudostratified columnar epithelium with oval nuclei located in the more basal aspect of the cytoplasm, and there may also often be presence of mucin [36,37].

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue was sectioned (3 μm), dried at 60°C, and processed in a Ventana BenchMark Ultra machine (Ventana Medical Systems Inc. (Tucson Arizona USA). Slides were incubated with monoclonal anti-COX-2 antibodies (Thermo Fischer Scientific rabbit), Universal Alkaline Phosphatase Red Detection Kit (Ultra View 760-501) and αSMA (Dako M.0851), DAB (Ultra View 760-500). Additional immunostaining on duplicates of twenty slides was performed with monoclonal COX-2 mouse antibody Invitrogen (Camarillo, CA, USA). Slides were counterstained with haematoxylin, fixed, mounted and analyzed using an inverted light microscope (Olympus, Center Valley, PA, USA).

Evaluation of COX-2 immunostaining

Immunohistochemistry was performed on whole tumour slices, which were assessed without prior knowledge of the clinical and pathological parameters. In each section, five different representative high-power fields (100×) with tumour infiltration were selected and examined by light microscopy. The intensity of staining was estimated on a scale from 1-3 (1-negative, 2-moderate, 3-strong). Cells were considered positive only if COX-2 intensity was moderate or strong. The extent of the immunolabeling was assessed as the percentage of positively stained tumour cells and was expressed on the scale from 1-3 where 1 represented less than 10% cells stained, 2 represented 10-50% and 3 over 50%. Since COX-2 demonstrated considerable heterogeneity within individual cases, the final immunoscore was obtained as the average of the numeric scores for five high-power fields of each case considered positive in intensity scoring. Based on histograms of the staining for all tumours, the optimal cut-off value for discrimination between negative and positive staining was found to be 1.4. Islets of Langerhans and mucosa of the duodenum were moderately to

strongly positive for COX-2, including those tumours with no COX-2 expression, and served as internal controls. Identical sections with omission of the primary antibody were used as negative controls. To test the validity of the Thermo antibody used for the study cohort, we performed additional immunostaining with a different monoclonal COX-2 mouse antibody, Invitrogen (Camarillo, CA, USA), on duplicates of twenty pancreatic cancer slides from the study cohort. The results were identical (Figure 1a and e). As Thermo antibody was not suitable for western blotting (producer recommendation), only the Invitrogen antibody was subjected to analysis by western blotting. The results showed a highly specific bond for COX-2 (Figure 1f).

Almost half of study specimens (44%) were evaluated independently by two examiners (EP and AS) and kappa interobserver was 0.73, indicating substantial agreement (95% CI 0.6-0.9).

Statistical analysis

Associations between variables were examined using Chi-square test, Fisher's exact test and Mann-Whitney test. Continuous variables were reported as median with corresponding range or interquartile range (IQR). Unadjusted survival analysis was performed using the Kaplan-Meier method, comparing curves using log-rank test. Multivariable Cox regression analysis was used for adjusted survival analysis. Possible interactions were evaluated by inclusion of an interaction term in the models. For all tests, a two-sided $p < 0.05$ was considered statistically significant. Statistical analyses were performed in SPSS 19 for Windows (SPSS Inc., Chicago, IL).

Results

The study cohort consisted of 230 patients consecutively resected for adenocarcinomas originating from the ampulla (AC) (n = 62, 27%), distal bile duct (DBC) (n = 76, 33%), or pancreas (PC) (n = 92, 40%). Median age at time of resection was similar for the three groups (67 years, range 37-83; $p = 0.463$ Kruskal-Wallis). Overall 5-year (actual) survival was 5% for PC, 16% for DBC, and 44% for AC ($p < 0.001$).

COX-2 expression and prognosis in ampullary, distal bile duct and pancreatic cancer

COX-2 staining was very similar in all three tumour types, with a positivity rate of 71% in PC, 72% in DBC, and 77% in AC. The COX-2 expression was detected in the cytoplasm of cancer cells in all three types of adenocarcinoma. No COX-2 immunostaining was detected in the stroma cells (Figure 1a,b, and e). The expression pattern showed heterogeneity both among different tumours and within the individual tumour, as areas with moderate to strong staining coexisted with negative areas within the same tumour (Figure 1c). Islet cells expressed moderately to strong COX-2 staining in all cases including those with no COX-2 expression in the tumour (Figure 1d). Irrespective of tumour origin, overall patient survival was more favourable in COX-2 positive than COX-2 negative tumours (Figure 2a-c). This was particularly prominent in AC ($p = 0.011$) and PC ($p = 0.043$) whereas the same tendency was seen in DBC although not reaching significance ($p = 0.06$). COX-2 expression varied according to the type of histological differentiation. In tumours with pancreatobiliary type of differentiation, two thirds of the tumours were COX-2 positive irrespective of anatomical origin (67%, 69%, and 68% in AC, DBC and PC, respectively). However there was no significant difference in overall survival when comparing COX-2 positive and negative tumours in this group (Figure 2d-f). All PC and DBC tumours with intestinal type of differentiation were COX-2 positive whereas 84% of the intestinal AC tumours expressed COX-2. The survival data of the intestinal AC tumours showed a favourable prognosis for patients with tumours expressing COX-2 ($p = 0.003$) (Figure 2g-i).

Factors associated with prognosis in pancreatic adenocarcinoma

COX-2 expression status was compared across clinical parameters associated with survival in the subgroup consisting of the 92 patients resected for pancreatic adenocarcinoma. The median survival for patients with COX-2 positive tumours was 18 months (95% CI 14-22) as compared to 11 months (95% CI 9.6-12) for patients with COX-2 negative tumours ($p = 0.043$). COX-2 positive tumours were more likely associated with high degree of differentiation ($p = 0.002$) and with intestinal type of differentiation, although, the latter did not reach significance ($p = 0.099$) (Table 1) probably due to the low number of tumours of the intestinal differentiation type.

There was no association with COX-2 positivity and R-status, lymph node ratio (LNR), lymph node status, tumour diameter, T classification, and vascular or perineural infiltration (Table 1). Since tumours expressing COX-2 were significantly more likely to be highly differentiated than COX-2 negative tumours, the joint effects of COX-2 status and differentiation grade on survival were assessed by Kaplan-Meier analysis, stratifying for COX-2 status (positive vs. negative) and differentiation grade (grade 1 and 2 vs. grade 3 and 4) (Figure 3a). Patients whose tumours did not express COX-2 and had a low differentiation grade (grade 3 and 4) had significantly poorer survival than the other three groups ($p = 0.006$).

In a previous report we found that LNR independently predicted prognosis in a multivariate model for survival

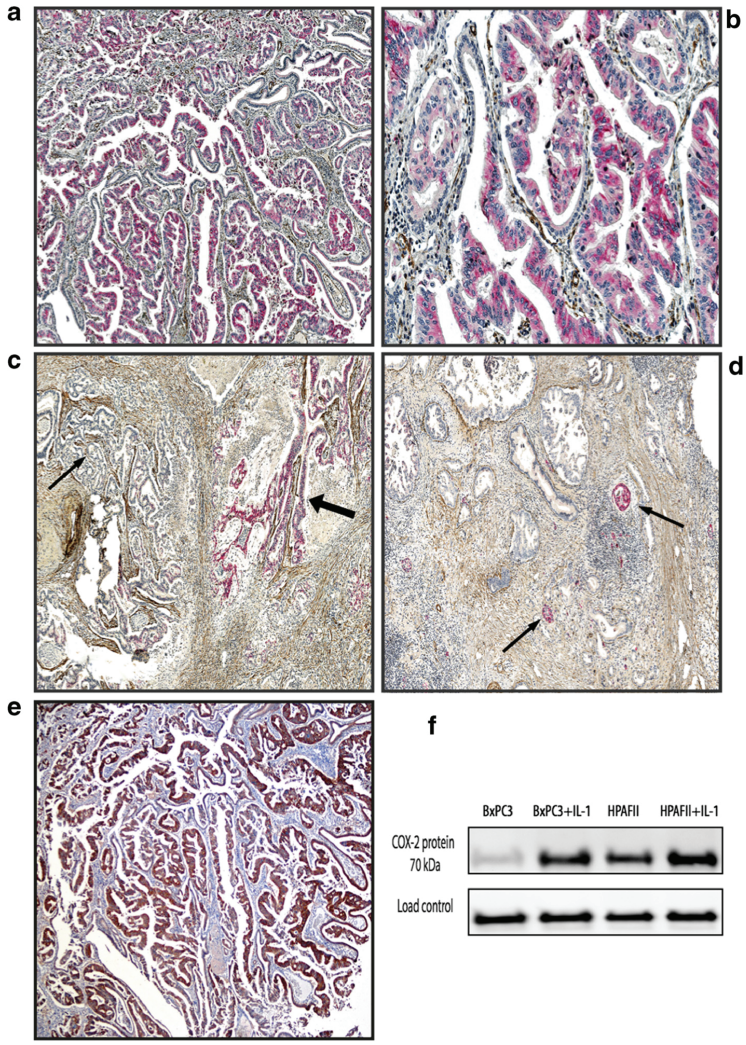

Figure 1 COX-2 expression in tumour tissue from pancreatic cancer. a-d Double immunostaing with monoclonal anti-COX-2 antibody (Thermo Fischer Scientific rabbit) and monoclonal anti-αSMA (Dako). COX-2 tumour positive cells (red colour), αSMA positive stromal cells (brown colour). **a** magnification × 100, **b** magnification × 200, **c** Heterogeneity in COX-2 expression within pancreatic cancer tissue. Areas with moderate to strong staining (thick arrow) coexist with COX-2 negative areas (thin arrow), (magnification x 100) **d** Moderately to strong COX-2 staining in islet cells (thin arrow), pancreatic cancer negative for COX-2 staining, (magnification x 100). **e** Immunohistochemistry of COX-2 expression in tumour tissue from pancreatic cancer. Immunostaining with monoclonal COX-2 mouse antibody Invitrogen (the same tumour as in **a**), magnification x 100. **f** Western blot of COX-2 expression in the moderately differentiated pancreatic cancer cell lines BxPC3 and HPAFII known to overexpress COX-2, with and without induction by interleukin 1 (Il-1), showed a specific bond for COX-2 (70 kDA) (monoclonal COX-2 mouse antibody Invitrogen).

in resected pancreatic cancer [38]. We thus also examined the joint effects of COX-2 status and LNR, and found that patients with COX-2 negative tumours and LNR >0.2 had significantly worst prognosis ($p < 0.001$) (Figure 3b).

In a multivariate analysis model including COX-2 expression, LNR, tumour size, margin status, vascular and perineural infiltration, COX-2 negative tumours and LNR > 0.2 independently predicted poor prognosis

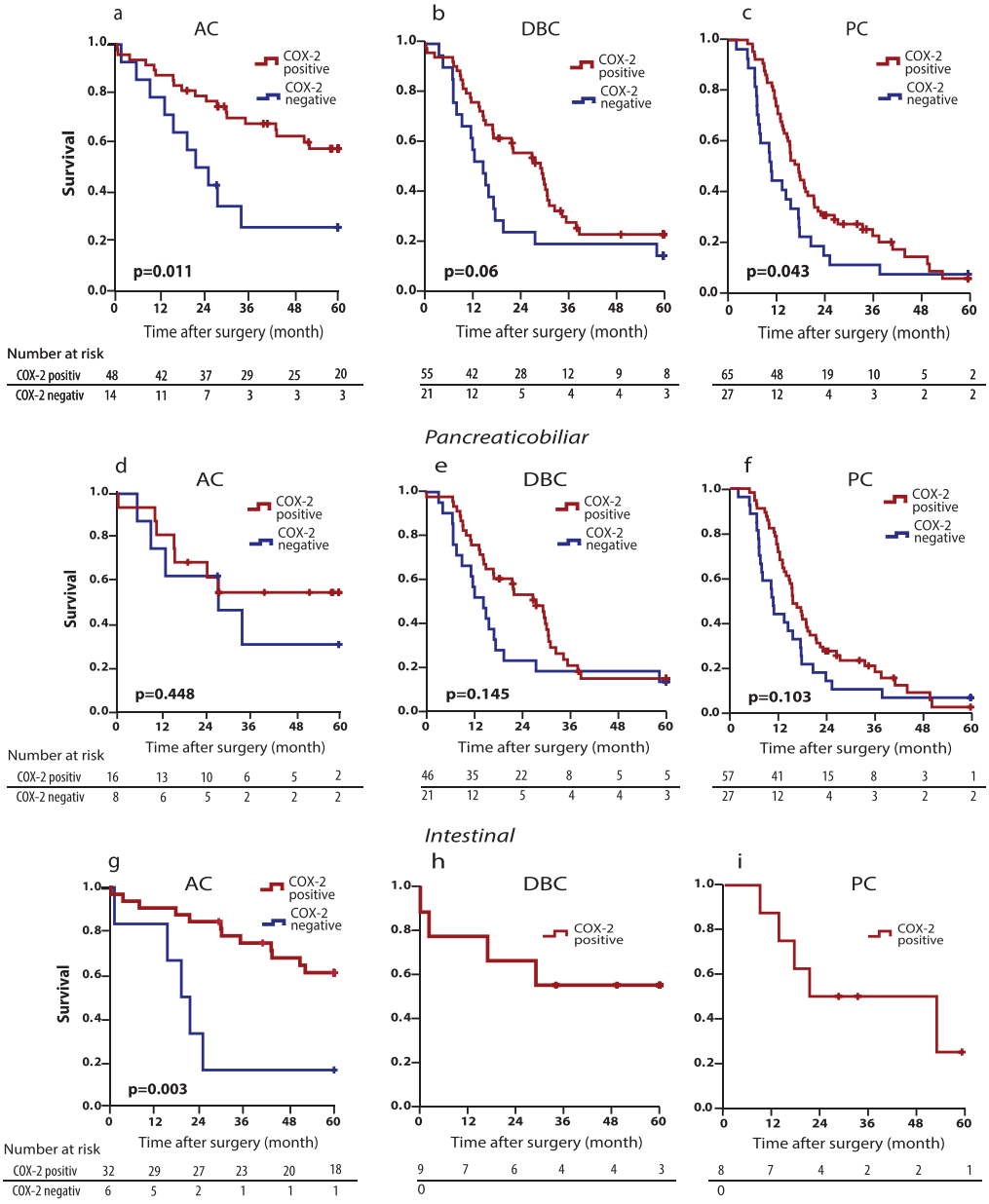

Figure 2 Overall survival analysis stratified by COX-2 expression. a Ampullary cancer (AC), **b** Distal bile duct cancer (DBC), **c** Pancreatic cancer (PC). **d**-**f** Overall survival analysis for AC, DBC and PC with pancreatobiliary differentiation stratified by COX-2 expression. **g**-**i** Overall survival analysis for AC, DBC and PC with intestinal differentiation stratified by COX-2 expression.

Table 1 Clinicopathological variables in 92 consecutive pancreatoduodenectomies for pancreatic cancer stratified by COX-2 status

Characteristic	n(%)	COX2-neg. n(%)	COX2-pos. n(%)	p[a]
COX-2				
Positive	65 (71%)			
Negative	27 (29%)			
Tumour size				
≤ 20 mm	15 (16%)	3 (20%)	12 (80%)	
> 20 mm	77 (84%)	24 (31%)	53 (69%)	0.54[b]
Lymph node metastasis				
N0, n (%)	25 (27%)	5 (20%)	20 (80%)	
N1, n (%)	67 (73%)	22 (33%)	45 (67%)	0.229
Lymph node ratio (LNR)[c]				
≤ 0.2	54 (59%)	13 (24%)	41 (76%)	
> 0.2	37 (41%)	13 (36%)	24 (65%)	0.251
Vascular invasion				
No, n (%)	30 (33%)	12 (40%)	18(60%)	
Yes, n (%)	62 (67%)	15 (24%)	47 (76%)	0.119
Perineural infiltration				
No, n (%)	15 (16%)	3 (20%)	12 (80%)	
Yes, n (%)	77 (84%)	24 (31%)	53 (69%)	0.54[b]
T classification				
T1	3 (3%)	1 (33%)	2 (67%)	
T2	6 (7%)	1 (17%)	5 (83%)	
T3	83 (90%)	25 (30%)	58 (70%)	0.851[b]
R1 resection status, n (%)				
R0, n (%)	40 (44%)	10 (25%)	30 (75%)	
R1, n (%)	52 (56%)	17 (33%)	35 (67%)	0.422
Degree of differentiation				
Grade I, II	53 (58%)	9 (17%)	44 (83%)	
Grade III, IV	39 (42%)	18 (46%)	21 (54%)	0.002
Type of differentiation				
Pancreaticobiliary, n (%)	84 (91%)	27 (32%)	57 (68%)	
Intestinal, n (%)	8 (9%)	0 (0%)	8 (100%)	0.099[b]

PC, pancreatic adenocarcinoma.
[a]Chi-square test, when not otherwise specified.
[b]Fisher's Exact Test.
[c]LNR assessment of 91 patients since in one specimen no lymph nodes were retrieved.

(Table 2). Since there was a strong correlation between COX-2 expression and differentiation grade ($p = 0.002$) it was not possible to include differentiation grade in the same model.

Only a minority of the patients received adjuvant chemotherapy. Although the numbers are small, there was no difference in survival between patients with COX-2 positive and COX-2 negative tumours who received adjuvant treatment.

Discussion

There is a large body of epidemiological, clinical and molecular evidence suggesting that COX-2 is implicated in the oncogenesis and progression of gastrointestinal malignancies, including adenocarcinomas derived from pancreatic head structures. It has previously been shown that COX-2 is upregulated in subsets of pancreatic, ampullary and distal bile duct adenocarcinomas although the proportion of upregulated tumours varies in the

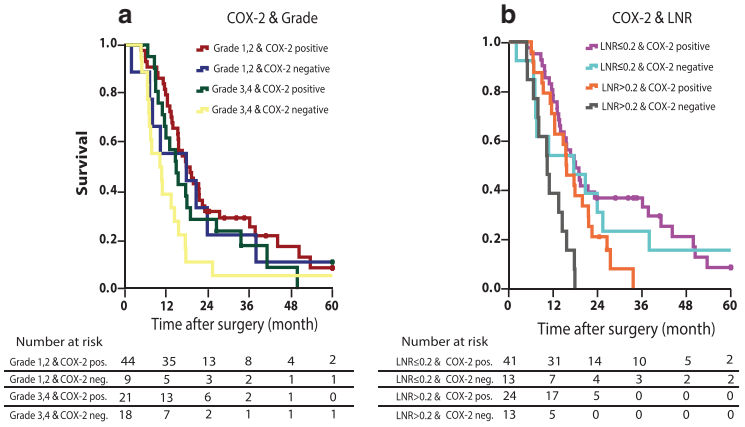

Figure 3 Overall survival analysis for patients with pancreatic cancer stratified by COX-2 expression and a degree of differentiation, b Lymph node ratio (LNR).

different reports. Furthermore, data on the prognostic importance of COX-2 expression in these tumours is conflicting. In pancreatic adenocarcinoma, two studies reported that COX-2 expressing tumours were associated with worse overall prognosis [19,21] whereas other studies have suggested a trend towards better prognosis for tumours with high COX-2 expression [22] or no association at all [39-41]. The present data on pancreatic, distal bile duct and ampullary adenocarcinomas indicates a more favourable overall survival for patients with COX-2 expressing tumours.

In periampullary and pancreatic head tumours, we have previously shown that histologic subtyping of these tumours into intestinal and pancreatobiliary types correlates with cell-type specific markers [36] and prognosis [3,37]. As COX-2 is thought to be expressed in epithelial cells throughout the gastrointestinal tract [5,12,42] it was of particular interest to examine whether there are differences in COX-2 expression in the intestinal and pancreatobiliary subtypes. Of note, most intestinal ampullary tumours (84%) were COX-2 positive, and in particular, all intestinal pancreatic and distal bile duct tumours were COX-2 positive. Patients with ampullary cancers of the intestinal subtype, which expressed COX-2, had a favourable prognosis with a 5-year actual survival of 60%. Histopathologic type of differentiation combined with biomarkers or gene expression profiles has recently attracted interest as important factors for outcome as well as stratification for adjuvant chemotherapy in ampullary adenocarcinoma [43,44].

Table 2 Multivariate Cox regression analysis of histopathologic factors in 92 patients with pancreatic cancer

	p-value	HR	95% CI
R-status (R1vs R0)	0.87	1.038	0.65 - 1.65
Vascular invasion (Involved vs non- involved)	0.455	1.208	0.74 - 1.98
Perineural infiltration (Involved vs non- involved)	0.359	1.369	0.70 - 2.68
Tumour size (> 20 mm vs ≤ 20 mm)	0.315	1.434	0.71 - 2.90
COX-2 expression (Negative vs Positive)	0.047	1.642	1.01 - 2.68
Lymph node ratio (LNR) (> 0.2 vs ≤ 0. 2)	0.032	1.757	1.05 - 2.94

The finding in the present study that COX-2 expression correlates with a favourable prognosis in pancreatic cancer can be explained by the fact that there is a statistically significant association between COX-2 positivity and high degree of differentiation. More than 80% of tumours with high differentiation grade showed overexpression of COX-2. This result is consistent with previous observations from studies of cultured pancreatic cancer cells and pancreatic cancer tissue. In cultured tumour cells COX-2 expression was found to be restricted to moderately and highly differentiated pancreatic cancer cell lines [23,26,45]. In human pancreatic adenocarcinoma tissue, well differentiated lesions expressed COX-2 to the highest degree, whereas there was less expression of COX-2 in moderately and poorly differentiated lesions [30]. In our study, the subgroup of patients with COX-2 positive/well differentiated tumours had a significantly better survival compared to patients with COX-2 negative/poorly differentiated tumours, whereas COX-2 positive/poor differentiation and COX-2 negative/high differentiation formed

an intermediate group with respect to survival. Thus the presence of COX-2 expression in these tumours appears to be a marker of favourable prognosis closely linked to the degree of tumour differentiation. Consistent with the latter the strong statistical association between COX-2 expression and differentiation grade precluded inclusion of both variables in the same multivariable model for survival.

The precise function of COX-2 in pancreatic cancer development is not known. In the normal pancreas, only islet cells always express COX-2 [24]. In transgenic mice models, overexpression of COX-2 in normal pancreatic ductal cells results in development of dysplastic changes resembling IPMNs and PanINs [46,47] suggesting a primary role of pancreatic cell COX-2 overexpression in the initiation of ductal adenocarcinoma. Recent evidence suggests that this is an intrinsic role of pancreatic cells independent of prostaglandins from the tumour microenvironment [48]. These observations support the concept that COX-2 overexpression might be a causal factor in pancreatic cancer development. It has also been suggested that pancreatic cancers that lack COX-2 (and COX-1) depends on exogenic prostaglandins from stromal fibroblasts for proliferation and other cancer-promoting effects [49]. Since COX-2 overexpression is implicated in tumour development, its expression in pancreatic cancer was hypothesized to result in a poor patient prognosis [19]. This hypothesis is difficult to reconcile with the observation that in fully developed tumours, COX-2 expression has been shown to be a function of differentiation status, with highest expression in well differentiated tumours [30]. In addition, it has been demonstrated that COX-2 expression varies markedly throughout the pathological process of pancreatic neoplasia. COX-2 expression increases in a stepwise manner with each initial stage of neoplastic progression up to the PanIN 2 stage, whereas COX-2 expression was relatively lower in invasive cancers [30].

Some of the discrepancies in results between our study and the studies by Juuti et al [19] and Matsubayashi et al [21] might be explained by methodological differences in patient sampling and/or tumour immunohistochemistry techniques. Since it is well known that it can be difficult to determine the precise anatomical origin of tumours of the pancreatic head, all cancers in the present series were re-evaluated for correct sub-classification into ampullary, distal bile duct or pancreatic tumours. There are also certain differences pertaining to the immunohistochemistry protocols that differ in our study compared to the studies by Juuti et al [19] and Matsubayashi et al [21]. In the work of Juuti, more than 30 years old specimens were included in the study cohort. It is known that for immunohistochemical staining protocols aging of fixed tumour tissue might interfere with staining [50]. Not only aging of the waxed specimen itself, but also variations in fixation protocols over time may result in inadequate staining. This may partly explain the low frequency of COX-2 staining (36%) in their data, compared to 55-80% in most other reports [20,22,23,25,26,41,51]. Since COX-2 expression in pancreatic tumours often is heterogeneous [24,29,30], the actual number of COX-2 positive tumours might be underestimated unless immunohistochemistry is performed on whole slide sections and assessed on multiple different high-power fields within each tumour. In the study of Matsubayashi [21], assessment of COX-2 staining was performed on tissue microarrays. Although this method has many advantages, tissue microarrays might not be the optimal method for assessment of COX-2 staining even if two cores of tumour tissues were studied from each tumour. This may partly explain the lower proportion of tumours expressing COX-2 in some studies [19,21] and hence the differences in patient survival.

Conclusion

COX-2 is overexpressed in pancreatic cancer, ampullary cancer and distal bile duct cancer and confers a survival benefit in all three cancer types. The overexpression is consistently linked to the histopathological type of differentiation and to the degree of differentiation. In pancreatic adenocarcinoma, COX-2 overexpression independently predicts a favourable prognosis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EP, OPC, IPG conceived and planned the study. EP and IPG conducted acquisition of data. EP, ARS, and OPC performed immunohistochemistry. EP, ARS, OPC and IPG analysed and discussed the results. EP and IPG drafted the manuscript. All authors critically revised and approved of the final manuscript.

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Author details

[1]Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway. [2]Department of Hepato-pancreato-biliary Surgery, Oslo University Hospital, Rikshospitalet, PO Box 4950, Nydalen, 0424 Oslo, Norway. [3]Department of Pathology, Oslo University Hospital, Rikshospitalet, Oslo, Norway.

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RESEARCH ARTICLE Open Access

Inhibitory effects of prostaglandin E_2 on collagen synthesis and cell proliferation in human stellate cells from pancreatic head adenocarcinoma

Ewa Pomianowska[1,2*†], Dagny Sandnes[3†], Krzysztof Grzyb[4], Aasa R Schjølberg[1], Monica Aasrum[3], Ingun H Tveteraas[3], Vegard Tjomsland[1,2], Thoralf Christoffersen[3] and Ivar P Gladhaug[1,2]

Abstract

Background: Several studies have described an increased cyclooxygenase-2 (COX-2) expression in pancreatic cancer, but the role of COX-2 in tumour development and progression is not clear. The aim of the present study was to examine expression of COX-2 in cancer cells and stromal cells in pancreatic cancer specimens, and to explore the role of PGE_2 in pancreatic stellate cell proliferation and collagen synthesis.

Methods: Immunohistochemistry and immunofluorescence was performed on slides from whole sections of tissue blocks using antibodies against COX-2 and α-smooth muscle actin (αSMA). Pancreatic stellate cells (PSC) were isolated from surgically resected tumour tissue by the outgrowth method. Cells were used between passages 4 and 8. Collagen synthesis was determined by [^{3}H]-proline incorporation, or by enzyme immunoassay measurement of collagen C-peptide. DNA synthesis was measured by incorporation of [^{3}H]-thymidine in DNA. Cyclic AMP (cAMP) was determined by radioimmunoassay. Collagen 1A1 mRNA was determined by RT-qPCR.

Results: Immunohistochemistry staining showed COX-2 in pancreatic carcinoma cells, but not in stromal cells. All tumours showed positive staining for αSMA in the fibrotic stroma. Cultured PSC expressed COX-2, which could be further induced by interleukin-1β (IL-1β), epidermal growth factor (EGF), thrombin, and PGE_2, but not by transforming growth factor-β1 (TGFβ). Indirect coculture with the adenocarcinoma cell line BxPC-3, but not HPAFII or Panc-1, induced COX-2 expression in PSC. Treatment of PSC with PGE_2 strongly stimulated cAMP accumulation, mediated by EP2 receptors, and also stimulated phosphorylation of extracellular signal-regulated kinase (ERK). Treatment of PSC with PGE_2 or forskolin suppressed both TGFβ-stimulated collagen synthesis and PDGF-stimulated DNA synthesis.

Conclusions: The present results show that COX-2 is mainly produced in carcinoma cells and suggest that the cancer cells are the main source of PGE_2 in pancreatic tumours. PGE_2 exerts a suppressive effect on proliferation and fibrogenesis in pancreatic stellate cells. These effects of PGE_2 are mediated by the cAMP pathway and suggest a role of EP2 receptors.

Keywords: Pancreatic stellate cells, Prostaglandin E_2, Cyclic AMP, DNA synthesis, Collagen synthesis

* Correspondence: ewa.pomianowska@medisin.uio.no
†Equal contributors
[1]Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway
[2]Department of Hepato-pancreato-biliary Surgery, Oslo University Hospital, Rikshospitalet, PO Box 4956, Nydalen 0424 Oslo, Norway
Full list of author information is available at the end of the article

Background

Pancreatic adenocarcinoma is one of the most lethal cancers of all solid malignancies with a 5 year survival of less than 5% [1-3]. A particular feature of primary pancreatic adenocarcinoma is the extensive fibrotic stromal reaction known as tumour desmoplasia surrounding these tumours [4-6]. There is increasing evidence that stromal cells are of major importance for tumour progression, by interacting in many ways with the malignant cells, such as reciprocal paracrine proliferative stimulation and angiogenesis, contributing to the early invasive growth and metastasis of this tumour [6]. These observations have raised the possibility that targeting the stromal cells to interrupt paracrine stromal signalling mechanisms may represent a new treatment strategy in pancreatic cancer. Animal studies have also indicated that targeting the tumour stroma of pancreatic cancer may improve drug delivery [7-9].

Multiple lines of evidence suggest that pancreatic stellate cells (PSC) have a major role in the development of pancreatic cancer desmoplasia [4-6,10]. These cells, which are normally quiescent cells in the pancreas, are induced during pancreatic injury to undergo transformation into a myofibroblast-like phenotype expressing alpha smooth muscle actin (αSMA). Studies of human and rat PSC in culture have identified a number of growth factors, cytokines, and hormones as regulators of pancreatic stellate cell activation [6]. Activation promotes PSC proliferation, migration, and extracellular matrix (ECM) deposition.

Overexpression of COX-2 has been reported in a number of epithelial cancers, including pancreatic cancer [11-16]. Transgenic mouse models have suggested that COX-2 overexpression in pancreatic ductal cells contributes to pancreatic tumour development [17,18]. Upregulation of COX-2 leads to increased production of prostaglandins, in particular PGE_2. PGE_2 may affect both cancer cells and different stromal cells through its effects on EP and FP receptors [19,20]. While EP2 and EP4 receptors are G_s-coupled receptors that stimulate adenylyl cyclase activity, EP3 receptors are G_i-coupled and inhibit adenylyl cyclase activity. EP1 receptors elevate the intracellular Ca^{2+}-levels through mechanisms that may involve both phospholipase C-dependent and independent mechanisms [19-21], and FP receptors are G_q-coupled and elevate intracellular Ca^{2+}-levels [19,20]. In addition, several of these receptors may signal via G protein-independent mechanisms [22].

Fibroblasts may be stimulated by PGE_2. Elevation of the intracellular level of cAMP in response to PGE_2 or other stimuli in fibroblasts from different tissues has been found to limit their proliferation, migration, and collagen secretion, as well as the differentiation of fibroblasts to myofibroblasts [23-25]. These effects appear to be mediated via EP2 and EP4 receptors. It has also been reported that PGE_2 may promote fibroblast proliferation through activation of EP1, EP3, or FP signalling [26-29]. In hepatic stellate cells, PGE_2 has been found to inhibit transforming growth factor β (TGFβ)-mediated induction of collagen mRNA [30], as well as proliferation induced by platelet-derived growth factor (PDGF) or thrombin [31,32]. However, the role of PGE_2 in pancreatic fibrosis is not well known. The aim of the present study was to examine further the effects of PGE_2 on pancreatic stellate cell proliferation and collagen synthesis.

Methods

Patients

The study protocol and patient consent documents were approved by the Regional Committee for Medical and Health Research Ethics (REC South East, project number S-05081), and was in compliance with the Helsinki Declaration. Written informed consent was obtained from all study participants. The study included only adults.

Chemicals

Dulbecco's Modified Eagle's Medium, Ham's F12 medium, RPMI 1640 medium, glutamine, and Pen-Strep (10.000 U/ml) were obtained from Lonza (Verviers, Belgium). HEPES, amphotericin, and heat-inactivated fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). Epidermal growth factor (EGF), adenosine 3':5'-cyclic monophosphate (cAMP), 3-isobutyl 1-methylxanthine (IBMX), L-ascorbic acid, and 3-aminopropionitrile fumarate salt were obtained from Sigma-Aldrich (St.Louis, MO, USA). Human platelet derived growth factor (PDGF), recombinant human transforming growth factor-β (TGF-β), and recombinant human interleukin-1β (IL-1β) were obtained from R&D Systems Europe, Ltd (Abingdon, England). Recombinant interleukin-1 receptor antagonist (Anakinra®) was a gift from Swedish Orphan Biovitrum AS, [6-^{3}H] thymidine (20–30 Ci/mmol), [2,8-^{3}H] adenosine 3',5'-cyclic phosphate ammonium salt (33.0 Ci/mmol), and L-[2,3-^{3}H] proline (55.0 Ci/mmol) were purchased from PerkinElmer (Boston, MA, USA). L161982 (N-[[4'-[[3-butyl-1,5-dihydro-5-oxo-1-[2-(trifluoromethyl) phenyl]-4 H-1,2,4-triazol-4-yl]methyl][1,1'-biphenyl]-2-yl] sulfonyl]-3-methyl-2-thiophenecarboxamide, AH6809 (6-isopropoxy-9-oxoxanthene-2carboxylic acid), and prostaglandin E_2 (PGE_2) were obtained from Cayman Chemical (Ann Arbor, MI, USA). Procollagen Type I C-peptide enzyme immunoassay kit was purchased from Takara Bio Inc., Japan. All other chemicals were of analytical quality. Antibodies against phosphorylated Akt^{Ser473}, total Akt, dually phosphorylated $ERK^{Thr202/Tyr204}$, and GAPDH were obtained from Cell Signaling Technology (Boston, MA, USA). Antibodies against COX-2 were obtained from Cayman Chemical (Ann Arbor, MI, USA)

or from Thermo Fischer Scientific Inc (Fremont, CA, USA). Anti-ERK antibody was from Upstate/Millipore (Billerica, MA, USA). Antibodies against TGF-β receptor II and PDGF receptor β were purchased from Cell Signaling Technology (Boston, MA, USA). Antibody against EP2 receptor was obtained from Cayman Chemical (Ann Arbor, MI, USA). Secondary antibodies were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Antibodies against vimentin and cytokeratins 7 and 19 were provided by DAKO (Glostrup, Denmark).

Isolation and culture of human pancreatic stellate cells

Human pancreatic stellate cells (PSC) were isolated by the outgrowth method developed by Bachem et al. [33]. Pancreatic tissue blocks (100–150 mg) were obtained during pancreatic surgery from patients with resectable pancreatic head adenocarcinoma. Altogether, stellate cell cultures were established from a total of 20 different patients. Briefly, the tissue blocks were cut using a razor blade (0.5–1 mm^3) and seeded in 10 cm^2 uncoated culture wells (6 per plate; 3–5 pieces per well) in a 1:1 (vol/vol) mixture of Dulbecco's modified Eagle medium (DMEM) with Ham's F12 medium, supplemented with l-glutamine (2 mmol/L), 100 U/ml Pen-Strep, 2.5 μg/ml amphotericin, and 10% FBS. Tissue blocks were cultured at 37°C in a 5% CO_2/air humidified atmosphere. Twenty-four hours after seeding, the small tissue blocks were transferred to new culture plates. Culture medium was changed every third day. The PSCs grew out from the tissue blocks 7 to 10 days later. The small tissue blocks were removed after 2–3 weeks. After reaching confluence, monolayers were trypsinized and passaged 1:3. The purity of the cells was assessed by morphology (most cells were stellate-like, with long cytoplasmatic extensions; some were also spindle shaped) and cytofilament staining of αSMA and vimentin. None of the cells were positive for cytokeratins 7 or 19 (data not shown). All experiments were performed using cell populations between passage 4 and 8.

Pancreatic adenocarcinoma cell lines

BxPC-3, HPAFII, and Panc-1 pancreatic adenocarcinoma cell lines were purchased from ATCC (Manassas, VA, USA). BxPC-3 cells were cultured in RPMI medium containing 4.5 g/l glucose, HPAFII cells were cultured in Dulbecco's modified Eagle's medium containing 1 g/l glucose, and Panc-1 cells were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose. The media were supplemented with glutamine (2 mM, or 4 mM in the case of Panc-1), 100 U/ml Pen-Strep, and 10% fetal bovine serum (FBS). Cells were plated in Transwell® inserts (Corning Incorporated, Corning, NY, USA) at a density of 100.000/cm^2 in serum-containing medium and cultured overnight. The next day, medium was replaced with fresh, serum-free medium, and cells were cultured overnight. The following day, the Transwells were transferred to 12 well Costar plates containing stellate cells in the lower compartment, and cells were cocultured for 48 hours.

Coculture of pancreatic stellate cells with pancreatic adenocarcinoma cell lines

Pancreatic stellate cells were plated at a density of 10.000 cells/cm^2 in 12 well Costar plates with serum-containing medium and cultured overnight. The following day, medium was replaced with fresh, serum-free medium, and cells were cultured overnight. The next day, the serum-free medium was changed, and Transwells containing pancreatic adenocarcinoma cell lines were placed on top. Cells were cocultured for 48 hours before harvesting for immunoblotting.

Measurement of DNA synthesis

Pancreatic stellate cells were seeded into 12 well Costar plates at a density of 10.000 cells/cm^2 in serum-containing medium and cultured overnight. On the following day, medium was replaced with fresh, serum-free medium. The next day, the serum-free medium was changed 30 minutes before addition of agonists. The cells were harvested after pulsing for 6 hours with [^{3}H]thymidine (18–24 hours after addition of agonists), and DNA synthesis was measured as the amount of radioactivity incorporated into DNA as previously described [34]. Briefly, medium was removed, and cells were washed twice with 0.9% NaCl. The cellular material was dissolved with 1 ml 0.5 N NaOH for 3 hours at 37°C, collected, mixed with 1 ml H_2O, and precipitated with 0.5 ml 50% trichloroacetic acid (TCA). The acid-precipitable material was transferred to glass fiber filters (GF/C Whatman, GE Healthcare, UK) and washed twice with 5.0 ml 5% TCA, followed by liquid scintillation counting of the filters in a Packard Tri-Carb 1900 TR liquid scintillation counter.

Measurement of collagen synthesis

Collagen synthesis was assessed by quantification of [^{3}H] proline incorporation into acetic acid-soluble proteins as described by Jaster et al. [35]. Pancreatic stellate cells were plated in 24 well Costar plates at a density of 10.000 cells/cm^2 in serum-containing medium and cultured overnight. The following day, medium was replaced with fresh, serum-free medium. The next day, serum-free medium was changed, and agonists and/or antagonist were added. After 24 hours, the medium was replaced with fresh serum-free medium containing 100 μg/ml ascorbic acid, 100 μg/ml 3-aminopropionitrile, and 2 μCi/ml [^{3}H] proline, and fresh agonists were added. The reaction was stopped 24 hours later, by addition of 50 μl/ml 10 N acetic acid. After an overnight incubation at 4°C, culture supernatants were transferred to microcentrifuge tubes,

mixed with 100 μl/ml FBS, 5 μg/ml rat tail collagen and 250 μl/ml 25% NaCl dissolved in 0.5 N acetic acid, and incubated at 4°C for 30 minutes. Protein precipitates collected by centrifugation (30 min, 10,000 g) were washed twice with 5% NaCl, followed by dissolution of the pellet in 0.5 N acetic acid. [^{3}H] proline incorporation was determined by liquid scintillation counting in a Packard Tri-Carb 1900 TR scintillation counter. In initial experiments, collagen synthesis was determined in parallell samples by measurement of procollagen type I C-peptide by an enzyme immunoassay. The two methods yielded similar results (data not shown).

RNA extraction and real-time quantitative RT-qPCR

Pancreatic stellate cells were plated at a density of 10.000/cm^2 in 20 cm^2 wells in serum-containing medium and cultured overnight. On the following day, medium was replaced with serum-free medium. The next day the medium was changed 30 minutes before agonists and/or antagonist were added, as indicated. The cells were stimulated for 24 hours. Total RNA was prepared from the samples using RNA Easy Mini kit (Qiagen Inc, Valencia, CA, USA) and cDNA was synthesized with SuperScript III Reverse Transcriptase First-Strand cDNA Synthesis kit according to the manufacturer's protocol (InVitrogen, Carlsbad, CA, USA). Quantitative PCR was performed with Platinum SYBR Green Master Mix (Life Technologies, Oslo, Norway) on 7900 Real-Time PCR system with 7900 System SDS 2.3 Software (Applied Biosystems) according to the manufacturer's protocol. Specific primers for collagen 1A1 were: forward, 5'-TGACGTGATCTGTGACG AGAC-3' and reverse, 5'- GGTTTCTTGGTCGGTGG GT –3' (Life Technologies Oslo, Norway). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized as housekeeping gene, and specific primers were: forward, 5'-CCACCATGGAGAAGGCTGGGGCTC-3' and reverse 5'-AGTGATGGCATGGACTGTGGTCAT3' (Life Technologies, Oslo, Norway). The primers were designed using Primer-BLAST [36]. All reactions were performed in triplicates including non-template controls. The results were analyzed using the ΔΔCt method [37]. Results for collagen 1A1 were normalized to GAPDH, and controls were assigned a value of 100%.

Cyclic AMP measurement

Pancreatic stellate cells were plated in 12 well Costar wells at a density of 10.000 cells/cm^2 in serum-containing medium. On the following day, medium was replaced with fresh, serum-free medium. The next day, medium was replaced with Krebs-Ringer-Hepes buffer, pH 7.4, containing 10 mM glucose. After preincubation for 30 minutes, cells were stimulated with PGE_2 or forskolin as indicated in the figure legends. The reaction was stopped by removing the buffer and adding 5% TCA. cAMP in the neutralized TCA extract was determined by radioimmunoassay as previously described [38].

Immunoblotting

Aliquots with approximately 7000 cells (total cell lysate prepared in Laemmli buffer) were electrophoresed on 12% (w/v) polyacrylamide gels (acrylamide: N'N'-bis-methylene acrylamide 30:1). This was followed by protein electrotransfer to nitrocellulose membranes and immunoblotting with antibodies against phospho-Akt, total Akt, phospho ERK1/2, total ERK, COX-2, and GAPDH, respectively. Immunoreactive bands were visualized with enhanced chemiluminescence using LumiGLO (KPL Protein research Products, Gaithersburg, MD, USA).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues from pancreatectomy specimens were sectioned (3 μm), and dried at 60°C. Further processing was carried out in the Ventana BenchMark Ultra machine (Ventana Medical Systems Inc. (Tucson Arizona USA) according to the manufacturer's recommendations. Slides were incubated with monoclonal anti-COX-2 antibodies (Thermo Fischer Scientific rabbit), Universal Alkaline Phosphatase Red Detection Kit (Ultra View 760–501) and a-SMA (Dako M.0851, DAB (Ultra View 760–500). Finally, slides were counterstained with haematoxylin, fixed, mounted and analyzed using an inverted light microscope (Olympus, Center Valley, PA, USA).

Immunofluorescence staining

Immunofluorescence staining was performed to examine COX-2 expression in the tumour slides. Formalin-fixed, paraffin-embedded tissues from pancreatectomy specimens were sectioned (3 μm), dried at 60°C and hydrated. Slides were incubated with monoclonal anti-COX-2 antibody (Thermo sp21 rabbit) and anti-αSMA (DAKO 1A4 mouse) for 30 min at room temperature in Ventana diluents. After washing with PBS, slides were incubated with secondary antibody conjugates (Alexa 555 anti-rabbit and Alexa 488 anti - mouse) in the dark for 1 hour in Dako diluents. After three washes with PBS, slides were mounted in VECTASHIELD containing DAPI (Vector Laboratories Inc., Burlingame, CA, USA). Fixed cells were observed under a fluorescence microscope.

Immunofluorescence staining was also performed on the cultured pancreatic stellate cells. Cells were first seeded into a Lab-Tek®II Chamber Slide™ System (Nunc International, Naperville, IL, USA) and were cultured for 24 hours before they were fixed in 4% paraformaldehyde at room temperature for 15 minutes. Cells were then washed three times and incubated with 5% BSA for 30 minutes to block non-specific binding. Slides were further processed as describe for tumour tissue.

Statistical analyses

Results are presented as mean ± standard error of the mean (S.E.M). DNA and collagen synthesis data were analyzed by one-way ANOVA, and post test using Bonferroni correction to compare groups, using GraphPad Prism (version 5.01, GraphPad Software, San Diego, CA, USA).

Results

COX-2 expression in pancreatic cancer cells

COX-2 expression in tumour tissue from pancreatic cancer was examined by double staining immunohistochemistry for COX-2 and αSMA. The cancer cells generally exhibited strong COX-2 staining (Figure 1A). We also found strong αSMA staining in the tumour stroma, indicating the presence of activated PSC. However, we could not detect double staining with COX-2 and αSMA in the stroma (Figure 1A). This was examined further by immunofluorescence, which failed to detect any COX-2 staining in the stroma (Figure 1B).

COX-2 expression in cultured human PSC

During culture of PSC, immunofluorescence staining in different passages revealed perinuclear staining with the COX-2 antibody in cells that were αSMA positive (Figure 1C). The expression of COX-2, αSMA, EP2 receptors, TGFβ receptors and PDGF receptors was found to be stable as a function of cell passage number as assessed by Western blotting (Figure 1D, 1E). Treatment of PSC with EGF and PGE_2 increased the expression of COX-2, whereas treatment with TGFβ did not. This expression pattern was observed in cells of both low and high passage numbers (Figure 1F). PDGF had no significant effect. Thrombin also induced COX-2 expression (data not shown). Interleukin-1β (IL-1β) was found to be a potent inducer of COX-2 expression, with maximal induction obtained at 0.1 ng/ml (Figure 1G). Coculture of pancreatic adenocarcinoma cell lines with pancreatic stellate cells was previously found to upregulate COX-2 mRNA in both stellate cells and adenocarcinoma cell lines [39]. We examined the effect of coculture of stellate cells with the adenocarcinoma cell lines BxPC-3, Panc-1, and HPAFII. Of these, only BxPC-3 cells induced COX-2 protein in the stellate cells (Figure 2A). Furthermore, this effect was abolished when the stellate cells were pretreated for one hour with an IL-1 receptor antagonist (Figure 2B).

PGE_2 stimulates EP2-mediated cAMP accumulation in PSC

PGE_2 may affect cells through both EP and FP receptors. Because fibroblasts from different tissues have been found to express mainly EP2 and EP4 receptors [24,40,41], we examined the effect of PGE_2 on cAMP accumulation in the stellate cells. When stellate cells were stimulated for 5 min with 100 μM PGE_2 or 50 μM forskolin, a direct activator of adenylyl cyclase [42], in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), cAMP levels were elevated 16.8 ± 5.8-fold (mean ± S.E.M.) above basal levels with PGE_2, and 33.0 ± 11.7-fold above basal with forskolin (n = 7). PGE_2 induced a strong, dose-dependent accumulation of cAMP, both in the absence and presence of IBMX (Figure 3A). When cells were pre-incubated with the EP4 receptor antagonist L-161982 [43], no significant inhibition of PGE_2-stimulated cAMP accumulation was observed. In contrast, AH6809, which is commonly used as en EP2 receptors antagonist [19], almost abolished the cAMP response, suggesting that cAMP accumulation in these cells is mediated mainly by EP2 receptors (Figure 3B).

PGE_2 inhibits DNA synthesis in PSC

We next examined how PGE_2 affected stellate cells proliferation. In agreement with previous studies [6,44,45], PDGF strongly stimulated DNA synthesis (Figure 4A). Epidermal growth factor (EGF) also stimulated DNA synthesis, although to a lesser extent than PDGF, whereas TGFβ had non-significant effect. (Figure 4A). In agreement with these findings, PDGF and EGF, but not TGFβ, significantly stimulated phosphorylation of both ERK and Akt in the stellate cells (Figure 4C). Interestingly, PGE_2, the FP selective receptor agonist fluprostenol, and thrombin also stimulated ERK phosphorylation in the stellate cells (Figure 4D), while they did not induce Akt phosphorylation (data not shown). The effect of PGE_2 and fluprostenol on ERK phosphorylation did not seem to involve cAMP, since forskolin did not stimulate ERK phosphorylation.

In human hepatic stellate cells several growth-stimulatory agents, including PDGF and thrombin, stimulate an acute PGE_2 production, as well as a delayed induction of COX-2, and pretreatment with a COX inhibitor enhances their growth stimulatory effect [31]. We examined the effect of pretreatment with indomethacin on PDGF-stimulated DNA synthesis in the pancreatic stellate cells. These experiments showed that pretreatment with indomethacin did not affect PDGF-stimulated DNA synthesis in the pancreatic stellate cells (Figure 4B).

Treating the stellate cells with PGE_2 did not significantly affect the basal DNA synthesis, but attenuated PDGF-stimulated DNA synthesis. PGE_2 exerted an inhibitory effect, which was significant at a concentration of 1 μM (Figure 5A). This effect was mimicked by forskolin (Figure 5B). cAMP levels were elevated above the basal level for at least 60 minutes following stimulation with PGE_2 (Figure 5C) or forskolin (Figure 5D). Neither fluprostenol nor thrombin had any effect on DNA synthesis, alone or in combination with PDGF (data not shown).

PGE_2 inhibits collagen synthesis in PSC

In agreement with previous findings [6,45,46] treating the stellate cells with TGFβ enhanced collagen synthesis,

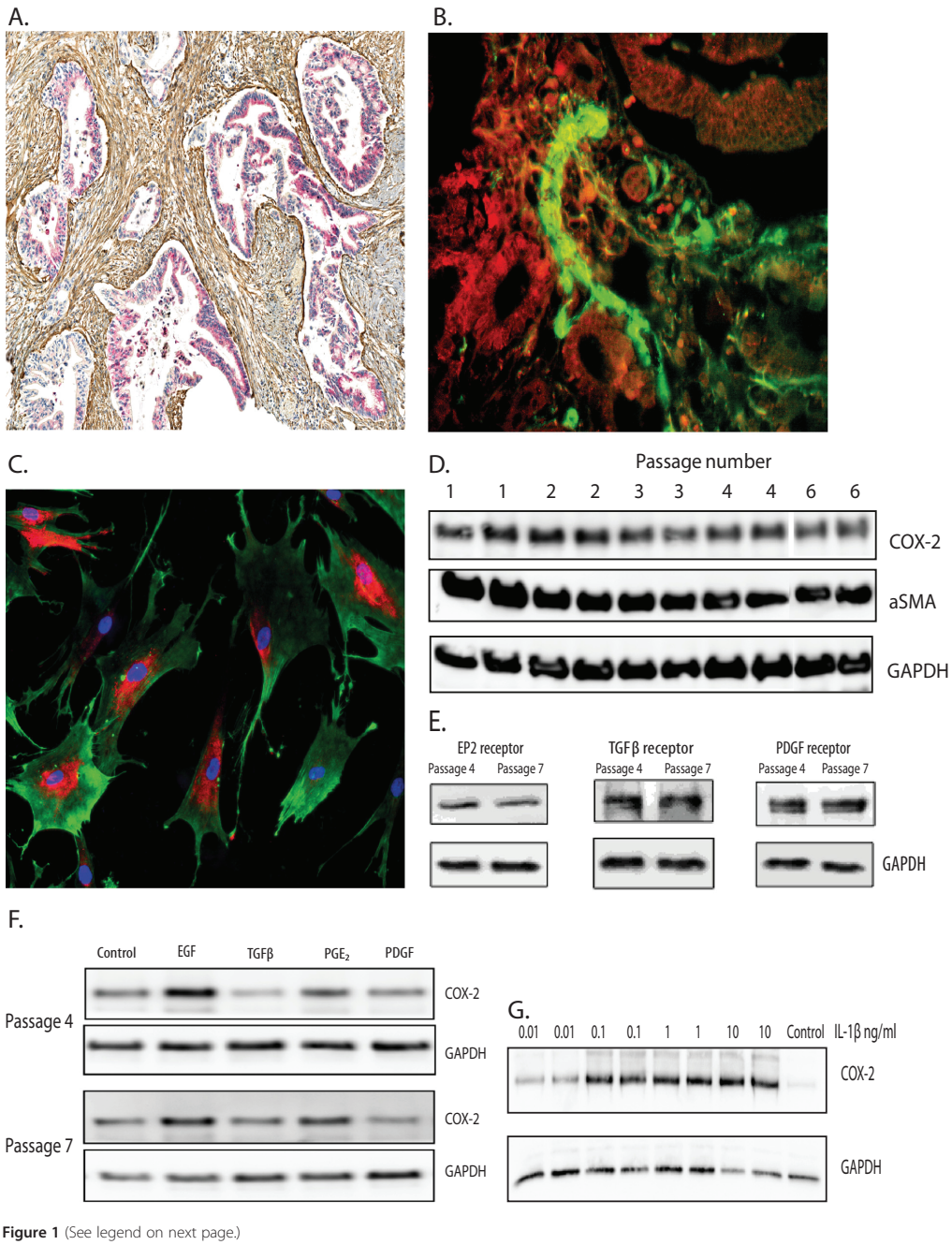

Figure 1 (See legend on next page.)

(See figure on previous page.)
Figure 1 COX-2 expression in formalin-fixed, paraffin-embedded tumour tissue from pancreatic cancer and isolated pancreatic stellate cells. A. Immunohistochemistry of COX-2 expression in tumour tissue from pancreatic cancer. COX-2 positive cells - red colour, fibrotic stroma αSMA positive - brown colour. **B.** Immunofluorescence of COX-2 expression in tumour tissue from pancreatic cancer. COX-2 positive cells - red colour, stroma αSMA positive - green colour. **C.** Immunofluorescence staining of cultured pancreatic stellate cells, passage five; COX-2 positive cells - red colour, αSMA positive cells - green colour, nucleus -blue colour. **D.** Expression of COX-2 and αSMA in different cell passage numbers. **E.** Expression of EP2 receptors, TGFβ receptors and PDGF receptors in two different cell passages **F.** Induction of COX-2 protein by EGF (10 nM), TGFβ (10 ng/ml), PGE_2 (10 uM), and PDGF (10 ng/ml) in two different cell passages. Cells in serum-free medium were stimulated with agonists for 24 hours before cells were harvested and lysates examined by Western blots as described in Methods. Results are from one representative experiment of four. **G.** Concentration dependent induction of COX-2 protein by IL-1β. Cells were stimulated in serum-free medium for 24 hours. Results are from one typical experiment of three.

whereas PDGF or EGF did not significantly affect collagen synthesis (Figure 6A). In agreement with the lack of induction of COX-2 by TGFβ in the stellate cells, pretreatment with indomethacin did not affect TGFβ-induced collagen synthesis (Figure 6B). Both PGE_2 and forskolin inhibited TGFβ-stimulated collagen synthesis, suggesting that this was a cAMP-mediated effect (Figure 7A, B, C). While we were preparing this manuscript, Charo et al. reported that PGE_2 stimulated the mRNA expression of collagen 1A1 in an immortalized human pancreatic stellate cell line [40]. To examine this further, RNA was extracted from cultured pancreatic stellate cells and assessed for elevated gene expression of collagen 1A1 by real time RT-qPCR. While TGFβ increased gene expression, PGE_2 alone showed a slight inhibitory effect, and significantly attenuated TGFβ-stimulated increase in gene expression of collagen 1A1 at a concentration of 1 μM (Figures 7D, 7E). Since PGE_2 might elevate cAMP levels through EP2 or EP4 receptors, we examined the effect of EP2 and EP4 receptor antagonists on collagen synthesis. We found that the EP4 receptor antagonist L161982 did not abrogate the effect of PGE_2 on TGFβ-induced collagen synthesis (Figure 7F) whereas results with the EP2 receptor antagonist AH 6809 were not conclusive (data not shown).

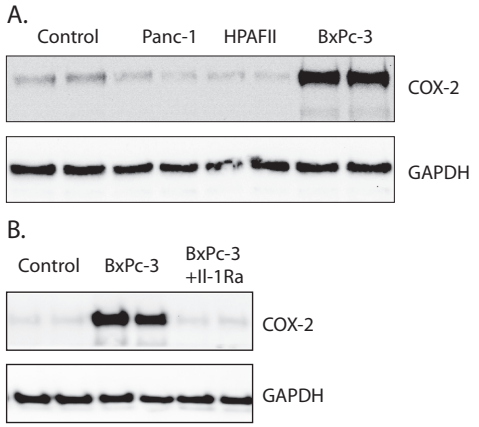

Figure 2 Induction of COX-2 protein in pancreatic stellate cells by indirect coculture with pancreatic adenocarcinoma cell lines. A. Effect of coculture with Panc-1, HPAFII, and BxPC-3 cells. Cells were cocultured in serum-free medium for 48 hours, before harvesting and analysis by Western blots as described in Methods. Results are from one typical of three experiments. **B.** Inhibition of COX-2 induction by coculture with BxPC-3 cells when stellate cells were pretreated with IL-1 receptor antagonist (1 μg/ml) for 1 hour before coculture for 48 hours. Results are from one typical of four experiments.

Discussion

In the present study we have demonstrated that PGE_2 inhibits both collagen and DNA synthesis in human pancreatic stellate cells from pancreatic adenocarcinoma. These effects are mediated by increased cAMP production. It is well known that in fibroblasts from lung and other tissues, PGE_2 inhibits proliferation by activating G_s-coupled EP2 and/or EP4 receptors [23-25,41,47,48]. Since EP4 inhibition affected neither the cAMP response nor the effect on collagen synthesis by PGE_2 in our study, it is most likely that EP2 receptors mediate these inhibitory effects of PGE_2 on cAMP and collagen synthesis. However, due to inconclusive results with the EP2 receptor antagonist, these mechanisms require further experimental confirmation.

In human hepatic stellate cells, thrombin and PDGF stimulate the release of PGE_2, which exerts an inhibitory effect on DNA synthesis induced by PDGF and thrombin [31]. However, PGE_2 appeared to mediate the mitogenic effect of EGF in BALB/c 3 T3 cells, and of PDGF in Swiss 3 T3 cells [49,50]. In our study, EGF, PGE_2 and thrombin, but not PDGF, consistently induced COX-2 protein expression in the stellate cells.

Pretreatment of the cells with indomethacin did not affect PDGF-stimulated DNA synthesis, suggesting that COX-2 induction and PGE_2 production neither mediated nor modulated PDGF-stimulated DNA synthesis. While we did not measure production of PGE_2, studies in various cells, including pancreatic stellate cells [40], indicate that levels are in the nanomolar range. We did not detect an effect of PGE_2 on DNA synthesis in the stellate cells when it was added alone, however, PGE_2, as well as forskolin, inhibited PDGF-stimulated DNA synthesis, suggesting that this effect was mediated by cAMP. This is in contrast

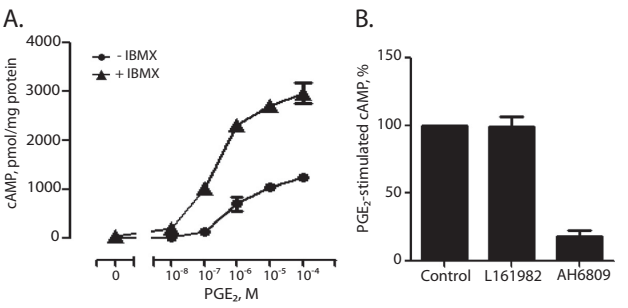

Figure 3 PGE_2-stimulated cAMP accumulation in pancreatic stellate cells. A. Dose-dependent effect of PGE_2 in the absence and presence of 0.5 mM isobutylmethylxanthine (IBMX). Cells were cultured as described in Methods, and were stimulated for 5 minutes. Results are presented as mean + S.E.M. of three replicates from one representative of three experiment. **B.** Effect of EP4 receptor antagonist (L161982, 10 μM) and EP2 receptor antagonist (AH6809, 30 μM) on PGE_2-stimulated cAMP accumulation (1 μM PGE_2). Cells were preincubated with antagonists for 30 minutes before stimulation with PGE_2 for 15 minutes in the presence of 0.5 mM IBMX. Results are presented as mean ± S.E.M. of five experiments.

to findings in rat pancreatic stellate cells, where treatment of the cells with conditioned medium from the Panc-1 adenocarcinoma cell line induced COX-2 expression and stimulated DNA synthesis [51]. Furthermore, inhibition of COX-2 activity with the COX-2 specific inhibitor NS-398 attenuated DNA synthesis in the rat stellate cells, albeit at high concentrations of the inhibitor, which may lead to nonspecific effects. Thus, at high concentrations of NS-398, inhibition of DNA synthesis has been reported in COX-2 expressing cell lines as well as in cell lines without COX-2 expression [52-54].

Pancreatic stellate cells are believed to be essential in the development of fibrosis associated with chronic pancreatitis and pancreatic cancer [4-6,10]. However, the role of PGE_2 in pancreatic fibrosis is unknown. TGFβ has been found to induce COX-2, which attenuates the profibrotic effect of TGFβ, in lung fibroblasts and hepatic stellate cells [30,48], and exogenous addition of PGE_2 inhibited TGFβ-induced collagen expression in hepatic stellate cells [30]. However, we found no induction of COX-2 by TGFβ in the pancreatic stellate cells, and preincubation of the cells with indomethacin did not affect TGFβ-stimulated collagen synthesis. In the lung, PGE_2 has been found to inhibit collagen synthesis by activating EP2 receptors and stimulating cAMP accumulation. In patients with idiopathic pulmonary fibrosis, lung fibroblasts display a diminished capacity to express COX-2 and to synthesize PGE_2. This results in decreased levels of PGE_2 and excessive fibroblast activation with massive fibrosis [41,47,48]. Our findings in the pancreatic stellate cells are consistent with these studies. Treatment with PGE_2, as well as forskolin, suppressed the increase in collagen synthesis stimulated by TGFβ, suggesting that this effect was mediated by cAMP. Our observations are thus in disagreement with findings in an immortalized human pancreatic stellate cell line, where 100 nM PGE_2 was found to induce mRNA of collagen 1A1 as well as other structural genes involved in extracellular matrix formation [40]. We therefore examined the effect of PGE_2 in our stellate cells, and found no evidence of collagen 1A1 mRNA induction. Rather, PGE_2 (1 μM) attenuated the TGFβ-induced expression of collagen 1A1, which is in agreement with our findings of an inhibitory effect of PGE_2 on collagen synthesis. The possibility that immortalized pancreatic stellate cells behave differently from primary cell lines needs consideration. Interestingly, the effects of PGE_2 on immortalized stellate cells were mediated by activation of EP4 receptors [40]. We have found no evidence of EP4 receptor involvement in the cAMP response in our primary stellate cells, however, we can presently not exclude the possibility that EP4 receptors signal via G protein-independent pathways [22].

We observed that PGE_2 stimulated ERK phosphorylation in the stellate cells. This effect was mimicked by thrombin and the FP selective agonist fluprostenol, but not by forskolin, suggesting that it was a cAMP-independent effect. Thus, the stellate cells may express other EP receptors or FP receptors that mediate this effect. PGE_2 has been reported to stimulate fibroblast proliferation through activation of EP1, EP3, or FP signalling in lung and cardiac fibroblasts, as well as in NIH 3 T3 cells [26-29]. If other prostaglandin receptors could stimulate proliferation of pancreatic stellate cells, the inhibitory effect of cAMP induced by EP2 receptors, appear to suppress these effects. It is notable that the inhibitory effect of PGE_2 on collagen and DNA synthesis was only significant at a concentration of 1 μM, whereas in lung fibroblasts effects have been observed at concentrations as low as 10 nM [41]. In a comparative study of fibroblasts from lung and gingiva, it was observed that stimulation with PGE_2 resulted in

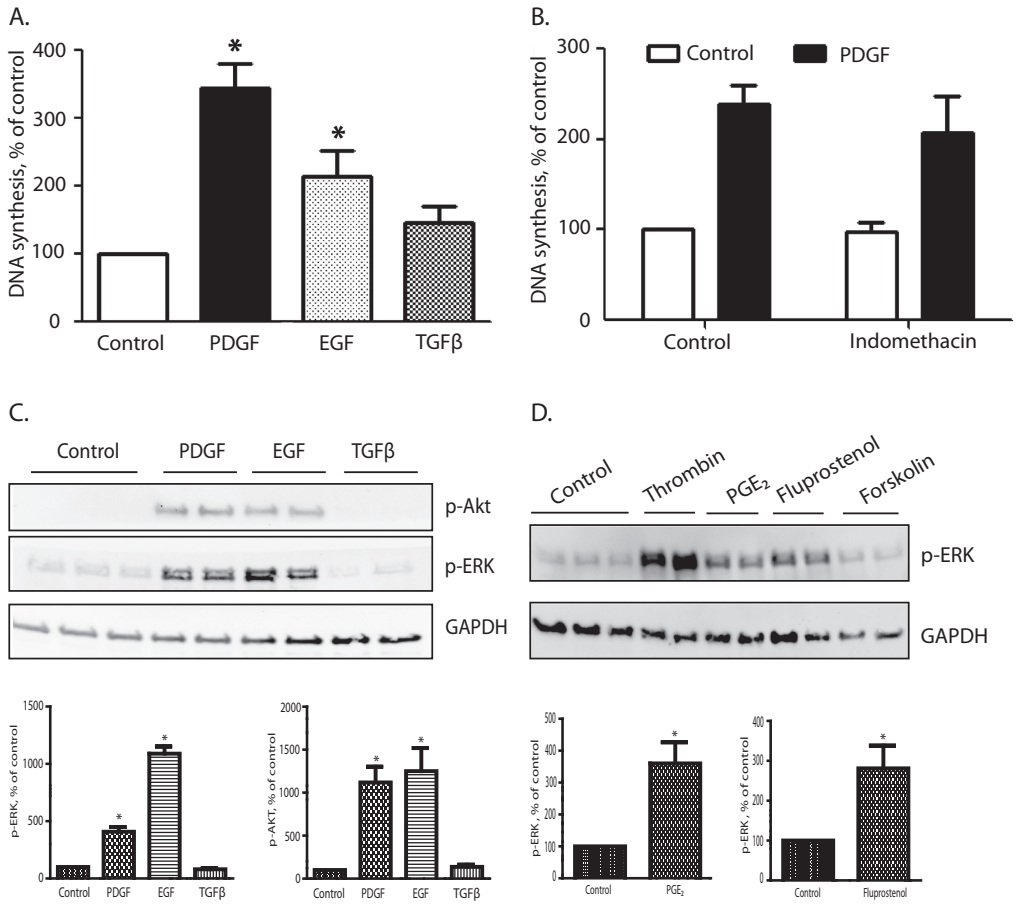

Figure 4 Effect of different agonists on DNA synthesis and phosphorylation of ERK and Akt in pancreatic stellate cells. A. Effect of PDGF (10 ng/ml), EGF (10 nM), and TGFβ (10 ng/ml) on DNA synthesis. Cells in serum-free medium were stimulated for 24 hours, with [^{3}H] thymidine added at 18 hours. DNA synthesis was assessed as described in Methods. Results are presented as mean +/−SEM of six experiments. **B.** Effect of pretreatment with indomethacin (10 μM) for one hour before stimulation of cells with PDGF for 24 hours. Results are presented as mean +/−SEM of three experiments. **C.** Effect of PDGF (10 ng/ml), EGF (10 nM), and TGFβ (10 ng/ml) on phosphorylation of Akt and ERK. Cells in serum-free medium were stimulated for 5 minutes before harvesting and analysis of cell lysates on Western blots. Blots are from one typical of four experiments. Histograms represent mean +/−SEM of four experiments. **D.** Effect of thrombin (1 U/ml), PGE_2 (10 μM), fluprostenol (10 μM), and forskolin (10 μM) on ERK phosphorylation. Cells were stimulated for 5 minutes before harvesting. Blots from one typical of four experiments are shown. Histograms represent mean +/−SEM of four experiments. * Sign. different from control.

less cAMP accumulation in gingival fibroblasts than in lung fibroblasts [55]. Furthermore, EP3 receptor activation induced phosphorylation of c-Jun NH_2-terminal kinase (JNK), which also mediated TGFβ-stimulated fibrosis. Thus, simultaneous EP3 receptor activation might reduce EP2-stimulated cAMP accumulation and blunt the inhibitory effect on DNA and collagen synthesis. Further studies, using subtype-specific agonists, or knockdown of prostaglandin receptors, are required to explore the role of other prostaglandin receptors on proliferation and fibrosis in the stellate cells.

Several previous studies have demonstrated that COX-2 is overexpressed in most human pancreatic cancers [12-16,56-60]. However, only a few publications have addressed COX-2 expression in pancreatic stellate cells and they reported no detectable COX-2 expression in the stroma [16,60]. In our study, immunohistochemical analysis carried out with a specific monoclonal antibody

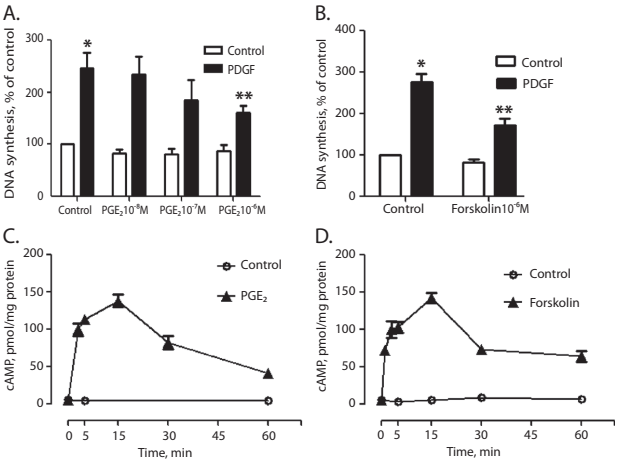

Figure 5 Effect of PGE_2 and forskolin on DNA synthesis and cAMP accumulation. A. Effect of increasing concentrations of PGE_2 on PDGF-stimulated DNA synthesis. Results are presented as mean ± S.E.M. of four experiments. **B.** Effect of 1 μM forskolin on PDGF- stimulated DNA synthesis. Results are presented as mean ± S.E.M. of four experiments. **C.** Time-dependent effect of 1 μM PGE_2 on cAMP accumulation in the absence of IBMX. Results are from one typical of four experiments and are presented as mean ± S.E.M. of triplicates. **D.** Time-dependent effect of 5 μM forskolin on cAMP accumulation in the absence of IBMX. Results are from one typical of four experiments and are presented as mean ± S.E.M. of triplicates. * Sign. different from control. ** Sign. different from PDGF alone.

revealed no detectable COX-2 expression in the stroma – neither in the normal pancreas nor in the pancreatic cancer. In contrast Charo et al. [40] reported COX-2 expression in the stroma. One reason for the discrepancy in the results could be the use of different antibodies. For immunohistochemical staining in the study presented by Charo [40] the polyclonal rabbit antihuman COX-2 antibody was used. It is known that polyclonal antibodies are more sensitive, but do not show as high specificity, as monoclonal antibodies [61]. To confirm the expression of COX-2 in pancreatic stroma, Charo at al [40] performed RT-PCR on isolated stellate cells. However, it is likely that the isolation process itself could cause activation of the stellate cells and increase the COX-2 expression [62].

Expression of COX-2 in cultured pancreatic stellate cells is well documented [40,51,63] and our results support these findings. In the immunofluorescence double staining of the cultured pancreatic stellate cells, only cells with positive expression for αSMA were additionally positive for COX-2. The COX-2 staining was perinuclear and

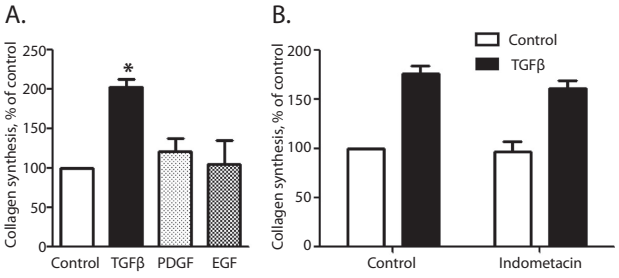

Figure 6 Effects of different agonists on collagen synthesis. A. Effect of TGFβ (10 ng/ml), PDGF (10 ng/ml) and EGF (10 nM) on collagen synthesis. Cells were cultured and stimulated with agonists for 48 hours, as described in Methods. [^{3}H] proline was present for the last 24 hours of stimulation. Collagen was precipitated and radioactivity in collagen was determined as described in Methods. Results are presented as mean ± S.E.M. of five experiments. **B.** Effect of pretreatment with indomethacin (10 μM) for one hour before stimulation of cells with TGFβ for 48 hours. Results are presented as mean ± S.E.M. of three experiments. * Significantly different from control.

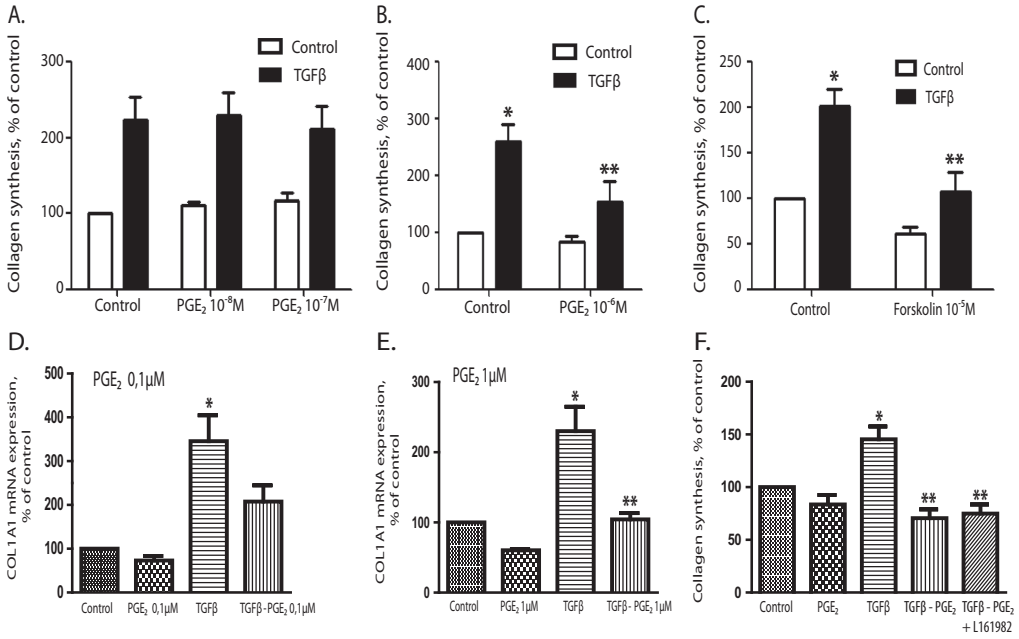

Figure 7 Effect of PGE2 and forskolin on TGFβ-stimulated collagen synthesis. A, B. Effects of different concentrations of PGE_2 on TGFβ-stimulated collagen synthesis. Cells were stimulated for 48 hours. Results are presented as mean ± S.E.M. of six experiments. **C.** Effect of forskolin on TGFβ stimulated collagen synthesis. Cells were stimulated for 48 hours. Results are presented as mean ± S.E.M of five experiments. **D.** Effect of TGFβ (10 ng/ml) alone and in combination with 100 nM PGE_2 on collagen 1A1 mRNA expression. Cells were stimulated for 24 hours before RNA was isolated and real time quantitative RT-qPCR was performed. Results are presented as mean ± S.E.M. of eight experiments. **E.** Effect of TGFβ (10 ng/ml) alone and in combination with 1 μM PGE2 on collagen 1A1 mRNA expression. Cells were stimulated for 24 hours before RNA was isolated and real time quantitative RT-qPCR was performed. Results are presented as mean + S.E.M. of three experiments. * Sign. different from control ** Sign. different from TGFβ alone. **F.** Effect of the EP4 receptor antagonist (L161982 1 μM) on PGE_2 influence on TGFβ-stimulated collagen synthesis. Cells were stimulated for 48 hours. Results are presented as mean ± S.E.M. of three experiments. *Sign. different from control ** Sign. different from TGFβ alone.

was constant in different passages (data not shown). COX-2 expression could be further induced by stimulating the stellate cells with IL-1β, EGF, thrombin, and PGE_2. Also, indirect coculture with the BxPC-3 cell line, but not HPAFII or Panc-1 cells, induced COX-2 expression. Pretreatment of the stellate cells with IL-1 receptor antagonist blocked the induction of COX-2 induced by BxPC-3 cells, which is consistent with the fact that the BxPC-3 cell line is known to produce IL-1α [64]. Interestingly, conditioned medium from Panc-1 cells induced COX-2 in rat pancreatic stellate cells, however, how this was mediated was not examined [51].

Conclusions

The present results show that COX-2 is mainly expressed in carcinoma cells, and suggest that the cancer cells are the main source of PGE_2 in pancreatic tumours. In the pancreatic stellate cells, PGE_2 exerts both antiproliferative and antifibrotic effects. These effects of PGE_2 are mediated by the cAMP pathway and suggests a role of EP2 receptors. Inhibition of COX-2 may inadvertently accelerate fibrosis progression in pancreatic cancer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EP, DS, TC, IPG conceived and planned the study. EP and KG isolated the pancreatic stellate cells. EP and DS did the cell culturing work. EP and ARS did the immunohistochemistry and immunofluorescence work. DS, EP, IHT, MA and VT conducted the experimental work. DS, EP and IPG analysed and discussed the results. DS, EP and IPG drafted the manuscript. All authors read and approved the final manuscript.

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Author details
[1]Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway. [2]Department of Hepato-pancreato-biliary Surgery, Oslo University Hospital, Rikshospitalet, PO Box 4956, Nydalen 0424 Oslo, Norway. [3]Department of Pharmacology, Faculty of Medicine, University of Oslo and Oslo University Hospital, Oslo, Norway. [4]Department of Pathology, Oslo University Hospital, Rikshospitalet, Oslo, Norway.

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